University of Alberta

Allelic diversity and tissue expression of MHC class I genes in the duck (*Anas platyrhynchos*)

by

Simona Mihaela Veniamin

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List of Abbreviations

AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cells
BiP	immunoglobulin binding protein
Bf	factor B
β_2-m	beta-2 microglobulin
C	core
CIITA	MHC class II transactivator
CD	clusters of differentiation
cDNA	complementary DNA
cccDNA	covalently closed circular DNA
CDR	complementarity determining region
CHIR	chicken immunoglobulin-like receptors
cim	class I modification
class I-a	classical MHC class I molecules
class I-b	non-classical MHC class I molecules
cln	calnexin
COUP-TFII	chicken ovalbumin upstream promoter-transcription factor II
crt	calreticulin
CTL	cytotoxic T lymphocyte
CTLR	C-type lectin-like receptor
D	diversity
DHBV	duck hepatitis B virus

EBV	Epstein-Barr virus
ER	endoplasmic reticulum
EST	expressed sequence tag
Fc	fragment crystallizable of immunoglobulin molecule
FCAR	receptor for IgA Fc
GPVI	platelet collagen receptor glycoprotein VI
GSHV	ground squirrel hepatitis B virus
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B endogenous antigen
HBV	hepatitis B virus
HC	MHC class I heavy chain
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSP	heat shock protein
HSV	herpes simplex virus
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
IFN	interferon
IL-2	interleukin 2
ILT	immunoglobulin-like transcript
IRF	interferon regulatory factor

ISGF3	interferon-stimulated growth factor 3
ISRE	interferon-stimulated response element
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
J	joining
KIR	killer cell immunoglobulin-like receptor
KSHV	Kaposi's sarcoma-associated herpes virus
LAIR	leukocyte-associated immunoglobulin-like receptor
LILR	leukocyte immunoglobulin-like receptors
LMP	low molecular weight protein
LRC	leukocyte Ig-like receptor complex
MAR	mouse activating receptor
MDV	Marek's disease virus
MHC	major histocompatibility complex
NF	nuclear factor
NK	natural killer
NKC	natural killer complex
NKLR	natural killer C-type lectin-like receptor
NOR	nucleolar organizer region
ORF	open reading frame
P	polymerase
PBR	peptide binding region
PCR	polymerase chain reaction

PIR	paired immunoglobulin-like receptors
Rfp-Y	restriction fragment pattern Y
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase PCR
STAT	signal-transducing activators of transcription
TAP	transporter associated with antigen processing
tapasin	TAP-associated glycoprotein
Th	T helper cell
TCR	T-cell receptor
TM	transmembrane
TNF	tumor necrosis factor
tpn	tapasin
3'UTR	3' untranslated region
V	variable
WHV	woodchuck hepatitis virus

1 INTRODUCTION

1.1 Major Histocompatibility Complex (MHC) class I

1.1.1 Structure of MHC class I molecule

MHC class I is a heterotrimer consisting of a 45 kD glycosylated heavy chain (HC), noncovalently associated with a 12 kD soluble protein, β_2-microglobulin (β_2-m), and a short peptide usually 8-10 amino acids long (Bjorkman et al., 1987; Saper et al., 1991) (Figure 1a). The heavy chain is a type I integral membrane protein with three amino-terminal domains, called α1, α2 and α3, a hydrophobic transmembrane domain and a carboxyl-terminal tail, that extends into the cytoplasm. The membrane distal domains, α1 and α2, combine to form the peptide binding region (PBR), consisting of two helices, one from each domain, resting on a sheet of eight antiparallel β-strands (Figure 1b). The PBR is closed at both ends. The membrane proximal domain, α3, has an immunoglobulin-like structure and interacts noncovalently with the other immunoglobulin-like domain of the molecule contributed by β_2-m. While both α3 and β_2-m support the β-sheet floor of the PBR, only α3 contacts the cytotoxic T-lymphocyte (CTL) bound CD8 glycoprotein (reviewed by Bjorkman and Parham, 1990). A conserved seven amino acid loop in the α3 domain serves as a binding site for the CD8 glycoprotein (Salter et al., 1990).

The PBR contains pockets that accommodate particular peptide side chains, termed anchor residues for their ability to anchor the peptide into the groove. These pockets, designated A through F, show a similar overall distribution in the MHC class I molecules (Matsumura et al., 1992). However, their shape and chemical nature vary between allelic variants and thus determine the set of peptides that can be bound by a

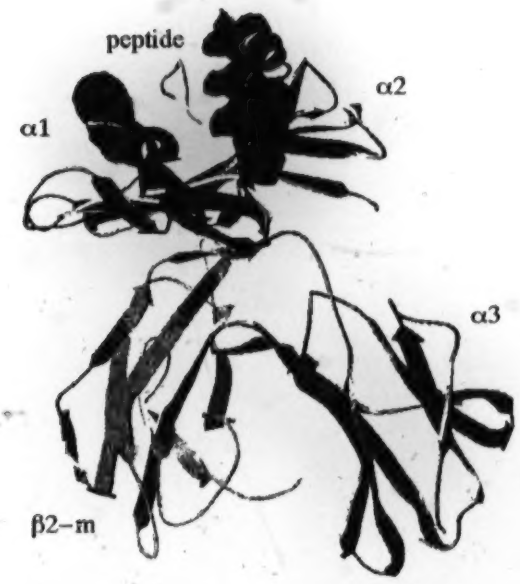

Figure 1a: The extracellular portion of the MHC class I molecule, formed by the α1, α2, and α3 domains of the heavy chain, β_2-microglobulin (light chain) and the bound peptide. Structure of the HLA-A2.1 molecule obtained from the Swiss-Prot entry. Image generated using the Swiss PdbViewer.

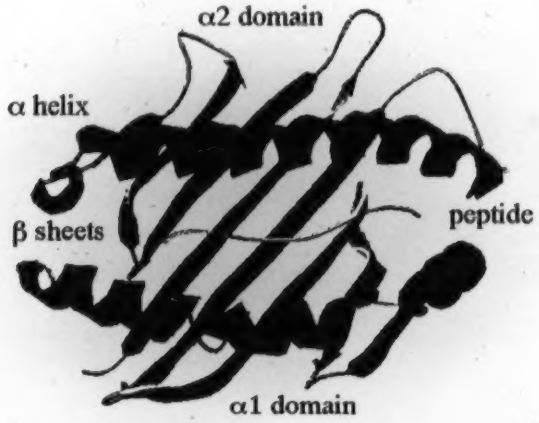

Figure 1b: Peptide binding region (PBR) formed by the α1 and α2 domains of the MHC class I molecule. Peptide lies in the groove in an extended conformation.

particular class I allele. Invariant residues in the two pockets located at opposite ends of the PBR, A and F, form multiple hydrogen bonds and ionic interactions with the free amino and carboxy termini of the peptide and therefore are essential for stable association and constrain the length of bound peptides to about 8-10 residues (Bouvier and Wiley, 1994). These conserved bonds between invariant residues in the PBR and structural components characteristic of all peptides account for the ability of class I molecules to bind a wide variety of peptide sequences. Over 2000 distinct peptides, present at 100-400 copies per cell, were identified in extracts from two different class I alleles (Engelhard et al., 1993). Additional hydrogen bonds are formed between residues in pocket F and the side chain of the amino acid at the C-terminal end. While the C-terminal residues of the peptides associated with human class I molecules are either hydrophobic or basic, the peptides bound by murine class I molecules have hydrophobic side chains at their C-termini (Gromme and Neefjes, 2002). The central pockets, B, C, D and E, interact with peptide side chains and allelic variation of the amino acids that form these pockets accounts for the ability of the MHC class I alleles to bind different sets of peptides. All peptides eluted from a particular class I allele show conservation at two or sometimes three residues, which are either identical or very similar in terms of size and polarity/hydrophobicity (Engelhard, 1994) (Figure 2). Those residues fit into the pockets of that particular MHC class I molecule and allow anchoring of all peptides of suitable length that contain them, irrespective of the amino acids at other positions. Thus each MHC class I molecule has a characteristic peptide binding motif (Falk et al., 1991).

Although the majority of peptides bound to MHC class I molecules are 8-10 amino acid long (typically nonamers) and lie into the groove in an extended conformation

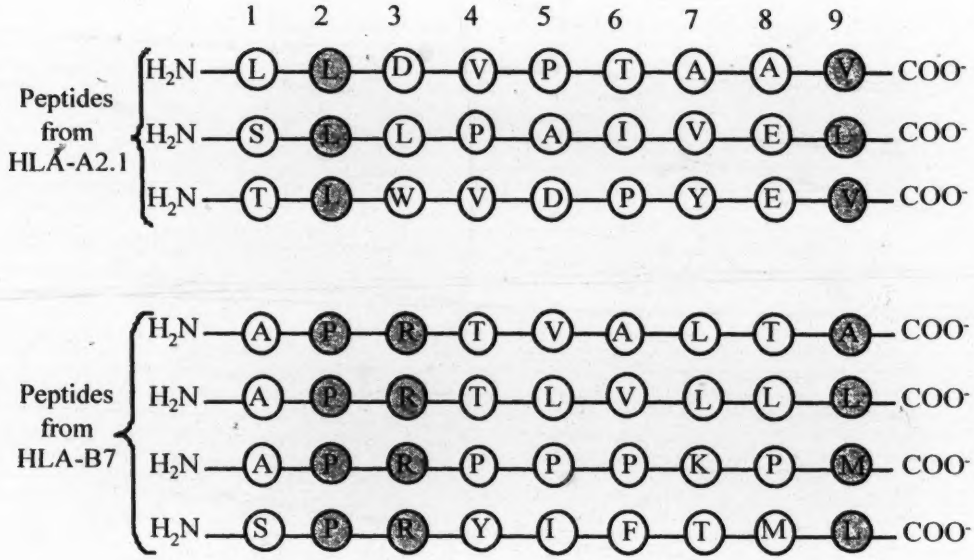
1 2 3 4 5 6 7 8 9
Peptides from HLA-A2.1
H2N L L D V P T A A V COO-
H2N S L L P A I V E L COO-
H2N T L W V D P Y E V COO-
Peptides from HLA-B7
H2N A P R T V A L T A COO-
H2N A P R T L V L L L COO-
H2N A P R P P P K P M COO-
H2N S P R Y I F T M L COO-

Figure 2: Peptides eluted from two human MHC class I molecules. The anchor residues are shaded. These residues are either identical or very similar in size and hydrophobicity for peptides that bind to a particular class I molecule. Adapted from Engelhard, 1994.

a few exceptions have been reported of peptides longer than 10 amino acids that can be accommodated in the PBR. Either the central portion of the peptide bulges out of the groove to allow for a correct anchoring of the N- and C-termini (Guo et al., 1992) or the C-terminal end of the peptide extends outside the PBR (Collins et al., 1994).

In all cases, the peptide-class I complexes are stable and support the idea that the peptide forms an integral part of the MHC class I structure, with an important role in assembly, stability and transport of MHC class I molecules to the cell surface.

1.1.2 Assembly of MHC class I molecules

MHC class I molecules are constitutively expressed on the surface of most but not all of the nucleated mammalian cell types (Daar et al., 1984). Their classical function is to bind peptides produced by the intracellular degradation of self or pathogen-derived proteins and display them on the cell surface for recognition by $CD8^+$ cytotoxic T lymphocytes (CTL) (Germain, 1994).

The assembly of the MHC class I trimer occurs in the endoplasmic reticulum (ER), where resident chaperones facilitate the proper folding and association of the subunits and retain them in the ER until assembly is correct and complete (Figure 3). The heavy chain (HC) is cotranslationally inserted into the ER membrane and, upon glycosylation, binds to a lectin-like chaperone with housekeeping functions, calnexin. This interaction appears to facilitate folding of the nascent HC and to promote assembly with β_2-m (Williams and Watts, 1995; Vassilakos et al., 1996). In β_2-m-deficient cells, the HC remains associated in the ER with calnexin, which may account for the inability of these cells to express significant amounts of class I molecules on their surface (Zamoyska and Parnes, 1988). However, normal assembly of MHC class I molecules in

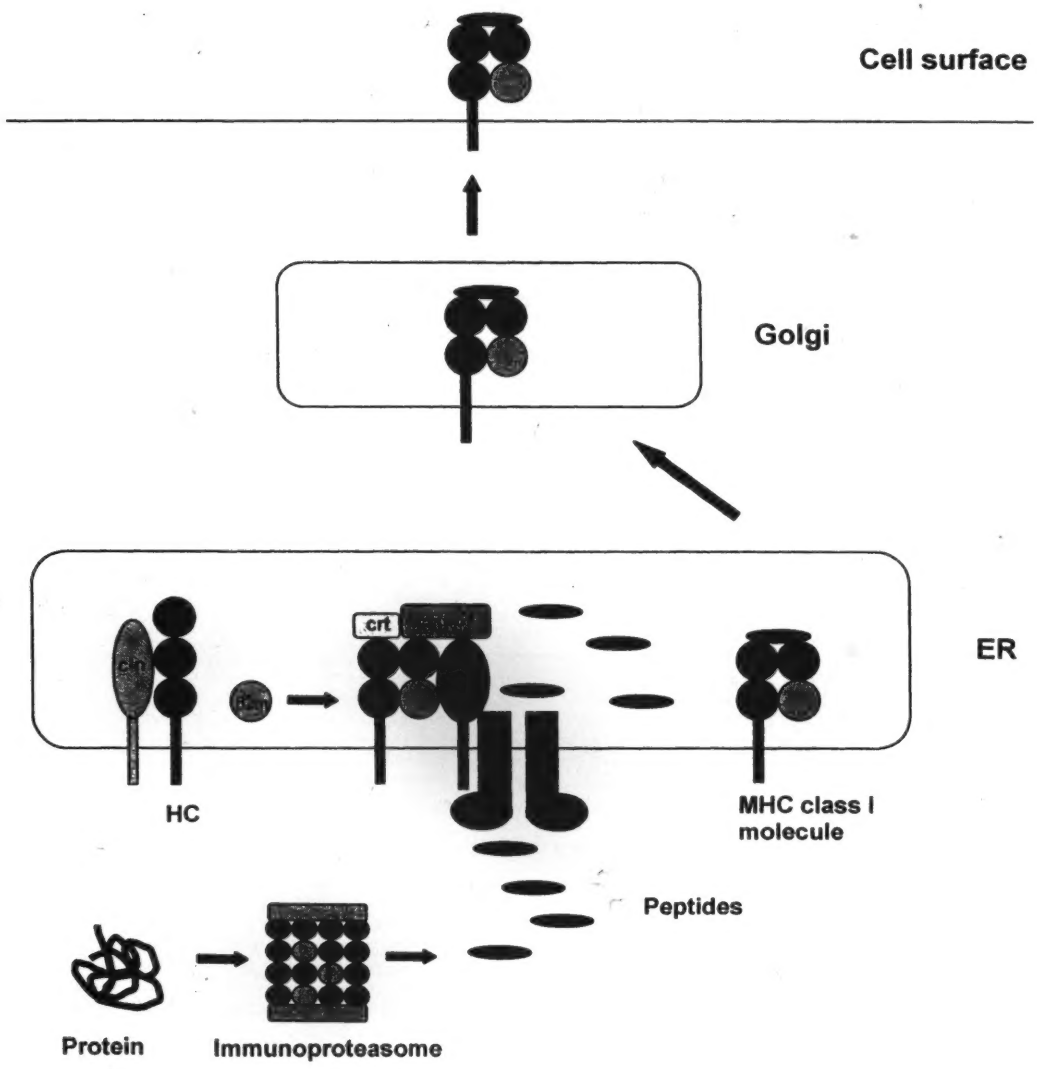

Figure 3: Assembly of MHC class I molecules occurs in the endoplasmic reticulum (ER), where resident chaperones facilitate the proper folding of the class I heavy chain (HC), its association with the β_2-microglobulin (β_2-m) and the loading of the heterodimer with peptides from the cytosol. MHC class I molecules reach the cell surface by the default secretory pathway. Abbreviations: cln, calnexin; crt, calreticulin; tpn, tapasin.

calnexin-deficient cell lines (Dawson and Scott, 1995) and evidence that another chaperone, the immunoglobulin binding protein (BiP), also binds free heavy chains (Nossner and Parham, 1995), supports the idea that calnexin is not an absolute requirement for proper folding of the HC. Following β_2-m binding to the HC, calnexin dissociates and the newly formed class I-β_2-m complex interacts with another lectin-like chaperone, calreticulin. The HC-β_2-m-calreticulin complex is then able to interact with the transporter associated with antigen processing (TAP), an interaction mediated by tapasin (TAP-associated glycoprotein) (Ortmann et al., 1994; Sadasivan et al., 1996). This allows formation of the MHC class I peptide-loading complex, consisting of the class I heterodimer, calreticulin, ERp57, tapasin and TAP. While calreticulin and ERp57 are ER chaperones with general housekeeping functions, tapasin and TAP are dedicated factors, critical for class I assembly. Tapasin not only brings the class I heterodimers in close proximity to TAP but also seems to influence the population of peptides that can bind in the class I groove, by catalyzing the replacement of low-affinity with high-affinity peptides (Androlewicz, 1999; Van Kaer, 2002). It has been proposed that tapasin stabilizes the 'empty' peptide-receptive state until binding of high affinity peptides induces a conformational change in the HC that closes the groove and leads to dissociation of class I molecules from the peptide-loading complex. Lower affinity peptides, less capable of inducing such a change, would be retained within the groove until loading with optimal ligands occurs. Thus, tapasin plays a role strikingly similar to that of the peptide exchange factor, DM, in the MHC class II pathway (Brocke et al., 2002). In tapasin-deficient cells, class I molecules fail to associate with the TAP

transporter, most of them are unstable and the few that can egress to the cell surface are bound predominantly by low-affinity peptides (Grandea III et al., 2000).

TAP, an integral ER membrane protein formed of two subunits, TAP1 and TAP2, translocates peptides from the cytosol into the ER lumen to be bound by the class I heterodimers (Kelly et al., 1992). Presence of TAP in the peptide-loading complex minimizes the distance peptides would have to diffuse in the ER to encounter an MHC class I molecule. The transporter, which functions in an ATP-dependent manner, shows both size and chemical selectivity. It efficiently binds peptides ranging from 8 to 16 residues in length, although longer sequences can be translocated with lower efficiency (Momburg et al., 1994a). Human TAP preferentially transports peptides with hydrophobic and basic C-termini, while the optimal C-terminal residue in peptides transported by murine TAP is hydrophobic (Momburg et al., 1994b). Therefore, the specificity of the transporter matches the chemical preferences shown by the F pockets of the class I molecules in these species, supporting the idea of coevolution of TAP and the linked MHC class I molecules for efficient cooperation in peptide presentation. In TAP-deficient cells, the peptide supply to the lumen of the ER is blocked, which makes the class I heterodimers unstable and leads to their translocation back into the cytosol for degradation by proteasomes (Hughes et al., 1997).

The peptides bound by the MHC class I molecules derive from cytosolic proteins, which are either at the end of their natural life or, most often, are newly synthesized. Between 30% and 80% of newly synthesized proteins are rapidly degraded providing a rich source of peptides for the antigen processing pathway and allowing the early detection of viruses or altered cells (Schubert et al., 2000; Reits et al., 2000). The large

diversity of peptides known to be presented by MHC class I molecules suggests that most, if not all, proteins available in the cell enter the antigen processing pathway, thus allowing for the presentation of as large a peptide repertoire as possible to include those newly synthesized (reviewed by Shastri et al., 2002). Upon ubiquitination, proteins enter the proteasome, which is the main proteolytic system in the cytosol of eukaryotic cells (Rock et al., 1994). This multimeric protease can cleave peptides at the C-terminal side of basic, hydrophobic, acidic, branched chain and small neutral aminoacids (Orlowski et al., 1993). Upon stimulation with interferon γ (IFNγ), a cytokine commonly produced after viral infection, three constitutive catalytic subunits are replaced by low molecular weight protein (LMP) 2, LMP7 and MECL1 in newly synthesized proteasomes (Fruh et al., 1994; Groettrup et al., 1996). This increases the peptidase activity after hydrophobic and basic residues while reducing cleavages after acidic residues and therefore leads to the generation of peptides that match the requirements for efficient transport by TAP and binding with high affinity to class I molecules. This way the immune system can use a general proteolytic pathway, designed to prevent the accumulation of prematurely terminated or misfolded proteins, to its advantage. The proteasomes containing the three interferon-inducible subunits are referred to as imunoproteasomes and represent the predominant form found in antigen presenting cells (APC) and inflamed tissues (Van Kaer, 2002). Immunoproteasomes generate mainly N-terminally extended precursors of the antigenic peptides, which have the C-terminal residues appropriate for translocation into the ER and binding with the class I molecules, but need further trimming at the N-terminus to fit into the binding groove (Cascio et al., 2001). While the generation of the C-terminus is restricted to cytoplasm, due to the absence of ER proteases capable of C-

terminal trimming (Eisenlohr et al., 1992), the N-terminal trimming can be done either by leucine aminopeptidase, the major trimming enzyme in the cytosol (Cascio et al., 2001), or, after translocation into the ER, by resident aminopeptidases (Elliott et al., 1995). Aminopeptidase trimming in the ER is a key step in the antigen processing pathway that generates a distinct set of peptides for class I molecules. This process, which results in the preferential accumulation of peptides with proline at position 2, explains the presence of the X-P-Xn motif among the MHC-bound peptide motifs (Barber et al., 1995), despite the inability of TAP to transport such peptides (van Endert et al., 1995). MHC class I molecules that bind the X-P-Xn motif might have evolved to take advantage of this unique pool of peptides generated by the aminopeptidases in the ER (Serwold et al., 2001). Presumably, these N-extended precursors are less susceptible to destruction by the endo- and aminopeptidases in the cytosol that degrade the majority of proteasomal products to amino acids for protein synthesis or for energy production. Although it is presently unknown how peptides to be presented by the class I molecules can avoid complete hydrolysis in the cytosol, it has been suggested that IFNγ-induced expression of leucine aminopeptidase favors peptide trimming over endoproteolytic cleavage of the epitope (Goldberg et al., 2002).

Peptides successfully translocated into the lumen of the ER, with the proper N- and C-termini, bind into the groove and induce the release of class I-β_2-m-peptide trimers from the peptide-loading complex. These completely assembled MHC class I molecules exit the ER by a selective mechanism involving association with transport receptors and move to the cell surface by the default secretory pathway (Spiliotis et al., 2000). Class I heterodimers that fail to form stable complexes with peptides fall apart in the ER and are

transported back into the cytosol, where they are subsequently degraded by the proteasome (Hughes et al., 1997). This way the erroneous loading of class I heterodimers with peptides in post-ER compartments is avoided. Likewise, peptides that did not bind with class I molecules exit the ER and become substrates for the proteasome. This retrograde transport is mediated in both cases by the Sec61 translocon, a system commonly used for the ER export of misfolded proteins and peptides (Pilon et al., 1997).

All the specialized proteins in the antigen processing and presentation pathway cooperate to ensure that stable complexes of class I heterodimers and high-affinity peptides reach the cell surface to interact with the CTLs. Complexes with low-affinity peptides, that could dissociate readily, would allow the pathogen in an uninfected cell to elude detection. In addition, peptide exchange between class I molecules from infected and uninfected cells would lead to misguided attack by the CTLs. Although very few, heterodimers with a loosely bound ligand may escape the editing mechanism and reach surface levels able to stimulate the CTLs or, after dissociation of peptide, give rise to empty class I molecules that allow exogenous peptides to sensitize a target cell (reviewed by Germain, 1999).

1.1.3 Expression of MHC class I molecules

Expression of MHC class I molecules is tightly regulated at the level of transcription. Constitutive and interferon-induced expression of class I heavy chain genes is controlled by transcription factors that bind to *cis*-acting regulatory elements of the promoter (van den Elsen et al., 1998; Gobin and van den Elsen, 1999). In addition to the CCAAT and TATA elements that bind and position the basal transcription initiation complex, the class I promoter presents two regulatory groups situated upstream of the

CCAAT box. One group, designated the S-X-Y module, contains the regulatory sequences, S, X1, X2 and Y, shared by MHC class I and class II genes (van den Elsen et al., 1998). This group includes binding sites for several transcription factors that mediate constitutive expression of class I genes such as RFX, which binds with X1, the ATF/CREB family of transcription factors, which binds with X2, and the NF-Y, which binds to the Y box (Gobin and van den Elsen, 1999). Upstream of the S-X-Y module, there is a group of regulatory sequences, absent in the MHC class II promoter. These include the enhancer A element, which controls constitutive transcription of class I genes, and the interferon-stimulated response element (ISRE), in charge of the interferon-induced transcription of class I genes (Gobin and van den Elsen, 1999). The enhancer A element contains binding sites for the transcription factors NF-κB and Sp1 (Gobin et al., 1998), while the ISRE element is responsible for binding factors of the interferon regulatory factor (IRF) family, such as IRF1 (Gobin et al., 1999).

In response to interferon α/β or γ stimulation, the phosphorylation cascade of the JAK/STAT signal transduction pathway is initiated (reviewed by Taniguchi et al, 2001). Type I interferons (IFNαs and IFNβ), produced by a variety of cells upon viral infection, and type II (IFNγ), produced by activated T lymphocytes and NK cells, show slightly different ways of activation of class I transcription. Binding of IFNα/β to its receptor leads to the phosphorylation of the signal-transducing activators of transcription (STAT) 1 and 2 in the cytosol and their association with the DNA binding protein p48 to form the interferon-stimulated growth factor 3 (ISGF3). Upon translocation into the nucleus, ISGF3 promotes class I transcription by binding the ISRE element. In addition, STAT1 homodimers induce the expression of the interferon regulatory factor (IRF) 1, which then

activates class I transcription by interacting with the ISRE element. IFNγ-induced activation of the phosphorylation cascade leads to the formation STAT1 homodimers. They can either complex with p48 to form a non-classical ISGF3, which then binds the ISRE or activate transcription of IRF1, which further interacts with ISRE.

Additionally, activation of the JAK/STAT pathway leads to the induced expression of the MHC class II trans-activator (CIITA), which can increase expression of the class I HC, presumably by interacting with the ATF/CREB transcription factors at X2 (Gobin et al, 1997). While the transcription of the MHC class II genes is completely dependent on CIITA, expression of the MHC class I promoters occurs in its absence. However, CIITA is required for optimal induction by IFNγ, as shown by a decrease of IFNγ-induced class I expression in dominant-negative CIITA cells (Martin et al., 1997). This activation route mediated by CIITA represents a direct link between IFNγ-induced MHC class I and class II gene regulation.

IFNγ enhances transcription of both class I HC and β_2-microglobulin and together with the tumor necrosis factor (TNF) causes a synergistic increase in steady-state mRNA levels and transcriptional rates of the class I heavy-chain and β_2-microglobulin genes (Johnson and Pober, 1990). In addition, IFNγ acts as a potent inducer of the expression of key players in the antigen processing and presentation pathway. By increasing expression of the LMP2 and LMP7 genes (Fruh et al., 1994) and MECL-1 gene (Groettrup et al., 1996) and incorporation of their products in the proteasome, IFNγ favors the production of peptides with C-termini best suited for binding with the class I molecules. In addition, it enhances expression of the TAP1 and TAP2 proteins and peptide transport capacity of the TAP (Ma et al., 1997). Expression of the tapasin gene and protein are strongly

induced by IFNγ and IFNβ and weakly induced by TNFα (Abarca-Heidemann et al, 2002). IFNγ-induced expression of leucine aminopeptidase might also promote class I presentation by favoring peptide trimming over endoproteolytic cleavage of the antigenic ligands generated in the cytosol (Goldberg et al., 2002).

1.1.4 Functions of MHC class I molecules

The main function of the classical MHC class I molecules (class I-a) is to present a selection of intracellularly derived peptides to $CD8^+$ cytotoxic T lymphocytes (CTLs). The interaction between the MHC class I molecule and the T-cell receptor (TCR) is critical early in ontogeny for the selection and control of a TCR repertoire capable of distinguishing between self and foreign antigens and also later on for the detection of cells that have been infected with intracellular pathogens or have undergone tumor transformation (reviewed by Benoist and Mathis, 1999). In addition, classical MHC class I molecules regulate the activity of the NK cells through interactions with the killer cell immunoglobulin-like receptors (KIR) and the C-type lectin-like receptors (CTLR) (reviewed by Sawicki et al., 2001).

Other MHC class I molecules, called non-classical and assigned to class I-b, perform functions that are not always related to the immune system. The non-classical molecules differ from the classical ones by their limited polymorphism, lower levels of expression at the cell surface, different tissue distribution and their ability to present nonprotein antigens to CTLs. In addition, some of the non-classical genes map outside the MHC region (Braud et al., 1999).

The following sections describe the immune and non-immune functions of MHC class I-a and class I-b molecules.

1.1.4.1 Functions of MHC class I-a molecules

MHC class I-a molecules bind intracellularly derived peptides that meet the requirements for length and presence of the proper anchor residues and display them on the cell surface. In a healthy cell, the peptides presented on the surface will be generated from self-proteins, while in a cell infected with a virus or an intracellular parasite, pathogen derived peptides will compete with self-peptides for the binding groove of class I molecules. Thus, there appears to be no specific mechanism for distinguishing between self and nonself at the level of peptide binding to MHC class I molecules and evidence suggests that this discrimination is done at the level of CTL, which upon recognition of the peptide-class I complex will trigger the adequate immune response (Bjorkman and Parham, 1990). An absolute requirement for CTL to perform its function is that the peptide be presented in the context of a self-MHC class I molecule and it is the complex formed by the two molecules that is recognized by the CTL receptor, which is said to be "MHC-restricted" (Zinkernagel and Doherty, 1974).

During ontogeny, T cells mature in the thymus from bone marrow derived precursors, which undergo a complex process of proliferation and selection assisted by thymic stromal cells. These cells, in addition to secreting soluble mediators of proliferation, express MHC class I and class II molecules upon which the immature T cells are educated. The T cell receptors (TCR) of immature T cells are exposed to class I and class II molecules associated with peptides derived from endogenous proteins and proteins originating from other cells that are taken up by the thymic stromal cells. Thus, immature T cells are screened during ontogeny for their ability to interact with self-MHC molecules associated with self-peptides expressed within the thymus. Immature T cells

expressing TCRs unable to bind any of the self-MHC-peptide complexes presented will be deleted. Too strong an association between a given TCR and a self-MHC-peptide complex will lead to the elimination of the cell bearing that receptor, and will thus prevent escape from thymus of a potentially autoreactive cell. This negative selection is the principal screen for establishing self-tolerance in the T cell repertoire. If the strength of the interaction between the TCR and self-MHC molecule is such that it does not stimulate cell activation but has the potential to activate the cell upon recognition of the same MHC molecule associated with a foreign peptide, then positive selection will occur. Thus, the TCR-self-MHC interaction is at the root of both negative and positive selection. The outcome of selection is apparently under the control of the TCR affinity for self-MHC-peptide complex and the overall avidity of the TCR for these ligands expressed on thymic stromal cells. Multiple TCR-self-MHC-peptide interactions are integrated to form a signaling gradient that defines cell fate. Numerous weak or limited high affinity interactions would trigger the maturation of the T cell, while a high number of high affinity interactions would lead to apoptosis (T lymphocyte selection reviewed by Benoist and Mathis, 1999).

Therefore, even if they cannot discriminate between self and foreign peptides at the level of binding, MHC class I molecules can shape a repertoire of T cells during ontogeny, that will be able later on to distinguish self from non-self in an MHC restricted manner. This role the MHC class I molecules, and class II alike, have in the selection of TCR is what limits the number of MHC loci expressed by an individual (Nowak et al., 1992). A higher number of different MHC molecules expressed would correlate with better chances to present pathogen-derived peptides and to trigger a cell-mediated

immune response. However, the more MHC molecules expressed, the more T cells deleted in the thymus to avoid autoreactivity and, consequently, a more reduced population of T-lymphocytes available for pathogen recognition.

Upon maturation in the thymus, two major T cell lineages are released to monitor the body for foreign antigens, αβT cells and γδT cells. They differ in their tissue distribution, type of antigens and the way they recognize them and the effector functions they perform. αβT cells, which account for the majority of T lymphocytes, recognize only peptide antigens associated with self-MHC molecules, while γδT cells recognize rather nonpeptide antigens in an MHC-independent manner, more reminiscent of an antibody-antigen interaction. The αβT cells can be subdivided into two subsets, depending on the co-receptor molecule that they express, CD4 or CD8. $CD8^+$αβT cells are primarily responsible for the elimination of cells infected by viruses or intracellular parasites, which they recognize in an MHC class I restricted manner. $CD4^+$αβT cells primarily regulate the function of other immune system cells, either by direct contact or by secreting mediators and they are MHC class II restricted. Based on the cytokines they produce, the $CD4^+$αβT cells can be further split into T helper (Th)1 cells, which secrete IFNγ and cytokines of the TNF family, therefore stimulating the cell-mediated immune responses, and Th2 cells, which produce cytokines that generally stimulate the antibody-mediated immune responses (reviewed by Benoist and Mathis, 1999).

A mature $CD8^+$αβT cell expresses a TCR consisting of two highly variable chains of the Immunoglobulin (Ig) superfamily, α and β, which contact both the antigen and the presenting MHC class I molecule, and of a complex of invariable chains, called CD3, which signals ligand engagement to the interior of the cell. During antigen recognition, a

co-receptor molecule, CD8, is recruited into preexisting TCR-peptide-MHC complexes, where it binds to the conserved class I α3 domain and stabilizes the TCR-ligand interactions. CD8 also brings the kinase Lck, constitutively bound to its cytoplasmic tail, into close proximity with the TCR and thus increase the sensitivity of T cells to antigen presented by class I molecules by about 100-fold. Signaling through the TCR and the CD8 co-receptor is not alone sufficient for T cell activation, which requires a co-stimulatory signal provided through engagement of the CD28 molecule on the T cell by the B7 molecules expressed on professional antigen-presenting cells (APC). Absence of the co-stimulatory signal prevents the activation of the T cell and induces a state of nonresponsiveness, called anergy, whereby the cell is no longer able of responding even when provided with both stimulatory and co-stimulatory signals (reviewed by Davis and Chien, 1999).

The α and β chains of the TCR, like those of immunoglobulins, present three classic hypervariable loops, termed the complementarity determining regions (CDR)1, 2 and 3. CDR1 and CDR2, which are encoded by V gene segments, show less variability than CDR3, formed by the random joining of V, D and J gene segments (Davis and Bjorkman, 1988). Crystal structure of the TCR-peptide-MHC class I complex revealed that the TCR is aligned diagonally over the peptide-MHC surface (Garboczi et al., 1996). CDR1 and CDR2 loops of the α chain contact the helices of the class I molecule and the conserved anchor residue at the amino terminus of the bound peptide while the CDR1 and CDR2 loops of the β chain interact with class I helices and the conserved anchor residue at the carboxy terminus of the bound peptide. The highly variable CDR3s of both α and β chains make contacts with the central amino acids of the peptide (Garcia et al.,

1996). Therefore, the skewing of diversity toward the CDR3 allows different TCR that recognize the same MHC class I molecule, through contacts between the less variable CDR1 and 2 and conserved amino acids in the helices, to differentiate between the various peptides bound by that molecule. This explains how a relatively limited number of different MHC class I alleles in an individual can select such a large repertoire of T cells. It also indicates that the sequence diversity in MHC class I molecules is primarily selected to increase their peptide-binding specificities and not to control interactions with TCR because most of the variable side chains are buried after peptide binding and thus not accessible to the TCR (Germain, 1999).

Upon recognition of the peptide-MHC class I complex by the TCR-CD3 complex, recruiting of the CD8 co-receptor and receiving of the co-stimulatory signal, intracellular signaling pathways are initiated. These lead to the activation of transcription factors that induce specific gene transcription that ultimately results in cell proliferation and differentiation and in cytokine secretion. The specific naïve $CD8^+\alpha\beta T$ cell, upon activation, will proliferate and differentiate into effector and memory $CD8^+\alpha\beta T$ cells. The effector cells will trigger the cytolysis of the infected cell, through Fas- or perforin-mediated mechanism, and will secrete proinflamatory cytokines, such as IFNγ, whereas memory $CD8^+\alpha\beta T$ cells will confer protection against re-infection with the same pathogen (reviewed by Benoist and Mathis, 1999).

MHC-TCR interaction is required not only during ontogeny in the thymus for shaping the repertoire of T cells but also for the survival of the mature T cells that patrol at the periphery. Naïve $CD8^+\alpha\beta T$ cells that do not encounter the specific antigen to trigger their expansion and differentiation will undergo apoptosis unless they receive a

survival signal from the MHC class I-restricting molecule. To survive they only need to interact with the restricting class I allele, to expand they also need the antigen. In contrast, memory $CD8^{+}\alpha\beta T$ cells can receive survival signals from any class I allele; however, for expansion, the restricting class I allele, but not the antigen is required. This suggests that memory $CD8^{+}\alpha\beta T$ cells might have a lower functional activation threshold that facilitates secondary responses (Tanchot et al., 1997).

Additionally, classical class I molecules interact with NK cell receptors of the KIR and CTLR families and regulate the activity of these cells. There are both activating and inhibitory isoforms within these receptor families, with similar extracellular domains, which suggests that they might recognize the same ligands. The intracellular domains differ, being short and devoid of signaling elements in the activating isoforms and long, with one or more immunoreceptor tyrosine-based inhibitory motifs (ITIM) in the inhibitory isoforms. The activating receptors associate with adaptor molecules that contain immunoreceptor tyrosine-based activation motifs (ITAM) in order to perform their function (reviewed by Bakker et al., 2000). The effector function of NK cells is regulated by a balance between signals delivered by the inhibitory and the activating receptors. As initially proposed by Kärre in the "missing-self" hypothesis, recognition of normal levels of MHC class I expression on the surface of somatic cells would inhibit NK cell activity (Kärre, 1985). Down regulation of MHC class I expression through viral infection or tumorigenic processes would result in a number of inhibitory receptor-MHC class I interactions insufficient to prevent NK cell activation. In the absence of the dominant inhibitory signal, the activating receptors can initiate NK cell activation and target cell lysis. Therefore, MHC class I molecules act as a link between the innate

immune response, represented by the NK cells, and the adaptive immune response, represented by the CTLs. These two types of effector cells are the components of a fail-safe system that makes possible the elimination of pathogens that would try to evade immune responses by either increasing MHC class I expression, to avoid NK cell recognition, or by decreasing MHC class I expression, to avoid T cell recognition (Yokoyama, 1999).

The physiological significance for the existence of both activating and inhibitory receptors for classical MHC class I molecules within the same family is still under investigation. Possible explanations for how the decision to engage an activating or inhibitory receptor is made have been proposed. The affinity of the activating receptors for the self-MHC class I molecules might not be sufficient to trigger effector function. Alternatively, the activating receptors might recognize ligands such as foreign antigens that resemble self-MHC molecules (reviewed by Cerwenka and Lanier, 2001).

1.1.4.2 Functions of MHC class I-b molecules

In humans there are several non-classical class I molecules encoded by genes that map within the MHC (HLA-E, -F, -G, HFE and MICA and MICB) or outside of this region (CD1 and the neonatal Fc receptor).

HLA-E binds peptides derived from the leader sequences of most classical class I molecules and of HLA-G and presents them to NK cells, which are thus able to monitor the integrity of the expression of the polymorphic class I molecules using a single receptor. Interaction between HLA-E and the inhibitory receptor CD94/NKG2A, a member of the CTLR family, on the surface of an NK cell will inhibit the cytotoxic activity of NK cells against targets that express normal levels of MHC class I molecules

and, consequently, of HLA-E (Borrego et al., 1998). Downregulation of MHC class I molecules occurring in virally infected or tumor cells will lead to the loss of surface expression of HLA-E, which will render the target cell incapable of providing inhibitory signals to the NK cells (Braud et al., 1999). Therefore, HLA-E allows the NK cells to check the integrity of cells and to avoid damage to normal tissues. The critical role of HLA-E in immunoregulation is emphasized by the finding that mouse expresses a homologue, Qa-1, with strikingly similar functions (DeCloux et al., 1997).

HLA-G is expressed on placental cytotrophoblast cells and on epithelial cells in the maternal thymus (Crisa et al., 1997). It has been detected in a membrane-bound form, which associates with β_2-m and endogenously derived peptides transported by TAP and also in a soluble form, which binds essentially the same set of peptides but does not seem to associate with TAP in the peptide-loading complex (Lee et al., 1995). HLA-G is the only class I molecule present on the surface of the trophoblasts and its low polymorphism might have been selected to prevent local activation of maternal alloreactive T cells and NK cells against the fetus. The fact that it is also expressed on the epithelial cells of the maternal thymus suggests that thymic selection events restricted by HLA-G may induce tolerance of the fetal graft presenting such HLA antigens and provide immunological protection of pregnancy (Crisa et al., 1997). A homologue of HLA-G in mice has not been detected, which implies a different placentation in the two species or the presence of a different molecule with a similar role (Braud et al., 1999).

The mouse H2-M3, with no equivalent in humans, is a non-classical class I molecule, which binds *N*-formylated peptides with much higher affinity than nonformylated ones (Wang et al., 1995). Since prokaryotic and mitochondrial ribosomes

initiate protein synthesis with formyl-methionine, H2-M3 might have evolved to present amino-terminal peptides from mitochondrial or bacterial proteins to CTLs and confer additional protection against intracellular bacteria.

MICA and MICB are heavily glycosylated proteins, which share low homology with MHC class I molecules (18%-30%) and display a relatively high level of polymorphism (Elsner et al., 2001). These non-classical class I molecules can activate NK cells, γδT cells and $CD8^{+}\alpha\beta$T cells through the NKG2D receptor (Steinle et al., 1998; Bauer et al., 1999). MICA does not associate with β_2-m, is conformationally stable in the absence of conventional class I peptide ligands and is almost exclusively expressed in the gastrointestinal epithelium (Groh et al., 1996). These and the presence of heat shock response elements similar to those of HSP70 genes in its promoter suggest that MICA might function as an indicator of cell stress and trigger the elimination of infected, damaged or transformed intestinal epithelial cells by the gut mucosal γδT cells or stimulate γδT cells to secrete growth factors for the maintenance of epithelial homeostasis (Groh et al., 1998).

HFE (formerly designated HLA-H) is expressed in association with β_2-m on cells in the intestinal tract and in the liver. It regulates the uptake of iron into the body through interactions with the transferrin receptor and individuals with a mutated HFE gene have an iron-storage disease, hemochromatosis, characterized by a massive iron overload in liver, pancreas and spleen (Gross et al., 1998; Roy et al., 1999).

CD1 molecules are encoded by non-polymorphic genes that map outside the MHC and even though they can present peptides to T cells, their binding groove is predominantly occupied by bacterial glycolipids (Sieling et al., 1995; Zeng et al., 1997).

Similar to classical class I molecules in their association with β_2-m, they differ with respect to peptide binding, which is TAP-independent (Teitell et al., 1997) and occurs in the acidified endosomal compartments, where peptides derived from extracellular proteins are loaded onto MHC class II (Sugita et al., 1999). CD1 targeting to endosomes is critical for presentation of bacterial glycolipids, which colocalize in these compartments (Prigozy et al., 1997). In addition, the presence of a narrower, deeper and highly hydrophobic binding groove is consistent with their preferential binding of lipid antigens (Zeng et al., 1997). Presentation of glycolipds by CD1 molecules to both $CD8^+\alpha\beta$T cells (Rosat et al., 1999) and $CD8^+\gamma\delta$T cells (Grant et al., 1999) diversifies the repertoire of antigens recognized by T cells and may strengthen the immune responses against bacterial infections. Binding of bacterial glycolipids, which are probably more difficult to alter than the peptide epitopes, might also explain the lack of polymorphism seen in the CD1 molecules (Kronenberg et al., 1999).

The neonatal Fc receptor, another class I-b molecule that maps outside the MHC, is an example of an MHC class I fold that was diverted to serve a different function, the interaction with the Fc portion of immunoglobulin. Its expression on murine neonatal intestine allows the uptake of IgG by the neonate, which is thus protected until it establishes its own immune system (Simister and Mostov, 1989).

1.1.5 Diversity of MHC class I molecules

The MHC function in immune responsiveness is reflected in its polygenic and polymorphic character. The presence of several different MHC class I and MHC class II genes within the complex (polygeny) and of multiple allelic variants of each gene within the population (polymorphism) makes it difficult for pathogens to evade immune

recognition. Moreover, the codominant expression of both alleles at a locus has the potential to double the number of different MHC molecules expressed in an individual and thus to increase the diversity already available through polygeny. In the human MHC, called HLA (Human Leukocyte Antigen), there have been identified 261 alleles at the HLA-A locus, 511 at HLA-B and 128 at HLA-C (Robinson et al., 2001). Given the high number of alleles and the relatively high frequency of each allele in the human population, the chance that the same allele be present at the MHC locus on both the homologous chromosomes of an individual is very low and thus most individuals will be heterozygous. The MHC loci are among the most polymorphic known, some of them with heterozygosities as high as 80% (Klein, 1986). This level of polymorphism, the self-MHC restriction of the T cell responses and the capacity of a large proportion of the T cell repertoire of any individual to respond to allogeneic MHC molecules (Lechler et al., 1990) account for the high frequency of rejection of transplanted tissues between individuals of the same species. Graft rejection, a rapid and very potent T cell-mediated immune response against transplanted tissues, can be triggered by as little as one amino acid difference between the MHC molecules of the donor and the recipient and it is much stronger than an immune response against a pathogen (Gould and Auchincloss, 1999).

The following sections present the molecular mechanisms that generate the diversity of the MHC class I molecules and the selective forces that maintain it.

1.1.5.1 *Mechanisms of generating diversity of the MHC class I molecules*

The majority of polymorphic positions in human MHC class I genes are clustered in the region encoding the $\alpha 1$ and $\alpha 2$ domains, which form the peptide binding groove. This region of the gene has a number of non-synonymous (amino-acid altering)

substitutions per site that greatly exceeds the number of synonymous substitutions, while in the rest of the gene the opposite is true (Hughes and Nei, 1988). The distribution and frequency of synonymous and non-synonymous substitutions indicate a positive selection for allelic diversity in the 5'part of the gene (exons 1 to 3) and for allelic homogenization and locus specificity in the 3' part (exons 4 to 8) (Parham et al., 1989). A comparison between the nucleotide sequences of class I alleles from the same locus showed that they are more similar to each other than to class I alleles from other loci. This suggests that genetic exchange between alleles of the same locus prevailed over genetic exchange events between alleles of different loci in the generation of class I gene diversity (Parham et al., 1988). Among the human class I genes, HLA-B seems to exhibit a uniquely high level of intra-locus (inter-allelic) recombination (McAdam et al., 1994), compared with HLA-A and HLA-C, in the case of which well-defined allelic lineages have persisted since prior to the human-chimpanzee divergence (Hughes et al., 1993; Yeager and Hughes, 1996).

Therefore, two main mechanisms seem to be involved in the generation of diversity among the MHC class I molecules, point mutation and genetic exchange between alleles at the same locus or at different loci. Variation is introduced into single alleles by point mutations, which are then propagated and assorted into new combinations through conventional meiotic recombination or through gene conversion (Parham et al, 1989). Recombination events, normally occurring during meiosis, allow for the exchange of nucleotide sequences between the alleles on the two aligned chromosomes of a pair. Inter-allelic recombination at the HLA loci can occur on both a large scale (involving exchange of one or more exons) and a small scale (involving exchange of short DNA

segments), the latter events showing a disproportionately high frequency in the region encoding the peptide binding groove (Hughes et al., 1993). Gene conversion, a process in which a DNA sequence from one chromosome is copied to the other, replacing the original sequence, might also occur during meiosis and might be favored by a misalignment of two homologous chromosomes that carry many copies of similar genes arrayed in tandem. The similarity and the close linkage of the HLA-A, B and C genes favor gene conversion events both between alleles of the same locus and between different loci. The predominance of locus-specific polymorphisms in exons 1 to 3 and of locus-specific substitutions in exons 4 to 8 suggests a higher frequency of conversion between alleles at the same locus than of conversion between alleles of different loci (Parham et al., 1989). However, this substitution pattern might result from selection and not from prevalence of a conversion event over the other. The lower level of similarity between class I loci makes it less likely that conversion between alleles at different loci will generate functionally useful products. Given the greater similarity between alleles of the same locus, the frequency of functionally useful products resulting from inter-allelic (intra-locus) conversion will be higher and selective amplification will occur (Parham et al., 1989).

The MHC class I diversity generated through point mutations and genetic recombination forms thus the ground on which selection can act. A low level of MHC class I polymorphism does not prevent the survival and expansion of a population, as is shown by the case of cheetahs and inbred mice, which live despite their paltry MHC. However, the entire population is potentially vulnerable to intracellular pathogens that subvert the immune response based on the common MHC class I type and that can trigger

catastrophic epidemics. A high level of MHC class I polymorphism makes it unlikely that all individuals in a population will be equally susceptible to a given pathogen and greatly enhances the chances of that population as a whole to survive in an environment with a large variety of intracellular pathogens. The benefit of MHC class I polymorphism is therefore quantitative and can only be felt at times of exposure to particular pathogens (Parham, 1999).

1.1.5.2 Selective forces that maintain the MHC class I diversity

There is a natural selection for MHC polymorphism and the proposed selective forces include resistance to infectious disease (pathogen-driven selection) and reproductive mechanisms that function to increase MHC heterozygosity (Potts and Slev, 1995).

Pathogen-driven selection can act through either frequency-dependent or over-dominant selection. The frequency-dependent selection model states that since most recognition evasion strategies are directed at hosts with common MHC genotypes, simply because pathogens spend more time in common hosts, pathogen evolution will disadvantage the common MHC alleles and advantage the rare ones. As the previously common alleles become more rare and the rare ones, common, the process repeats itself, leading to a frequency-dependent cycle, able to maintain a large number of alleles (Takahata and Nei, 1990; Slade and McCallum, 1992). The ability of this process to maintain genetic diversity is critically dependent on the lag between the time an MHC allele becomes rare, because it does not confer resistance, and the time it gains advantage due to pathogen-escape events directed toward the common alleles (Potts and Slev, 1995). An example of this type of selection in humans is the increased frequency of the

HLA-Bw53 allele in West Africans, compared with other racial groups. Presence of this allele is associated with protection from severe malaria and its frequency in the West Africans accounts for as great a reduction in the disease incidence as the sickle-cell haemoglobin variant (Hill et al., 1991).

In the over-dominant selection model, heterozygote individuals are selected due to their advantage over the homozygotes in fighting pathogens. Since heterozygotes can present a larger array of antigenic peptides than the homozygotes, it takes longer for escape mutants to arise in heterozygote than in homozygote individuals. Additionally, because resistance is dominant over susceptibility (Schwartz, 1986), in a heterozygote the resistance profiles of both haplotypes will manifest, while the susceptibility profile of each haplotype will be masked. Heterozygosity will confer an advantage in all cases, except when both alleles associate with susceptibility. An example of this type of selection in humans is the delayed onset of acquired immunodeficiency syndrome (AIDS) in patients infected with human immunodeficiency virus-type 1 (HIV-1), who present maximum HLA heterozygosity of class I loci. Individuals homozygous for one or more loci show a more rapid progression of the infection to AIDS and death (Carrington et al., 1999).

Reproductive mechanisms that favor MHC polymorphism include mating preferences based on MHC-influenced odors (sexual selection), avoidance of inbreeding and selective fertilization and abortion. Studies conducted in humans showed that mate selection could be dependent on HLA differences, detected through HLA-influenced body odors. Females and males, typed for their HLA alleles, when asked to score the odors of worn T-shirts showed preference for individuals with a more dissimilar HLA set

of alleles (Wedekind et al., 1995; Wedekind and Furi, 1997). The results of the test suggest that mate choice could be influenced in a way that preferences function to increase heterozygosity, and potentially immunocompetence, in the offspring. A subsequent study showed that females can detect differences of one HLA allele among male odor donors and that they prefer mates with whom they share one or two alleles (Jacob et al., 2002). The alleles common to the female recipient and the male donor were inherited by the recipient paternally, suggesting that a woman's odor choice is associated with inherited HLA alleles rather than with exposure to HLA-associated odors from her family during development. This study also showed that a small, intermediate number of HLA matches is preferred over no matches or similar HLA. The contrasting results obtained in the two studies can be explained when taking into consideration that Wedekind et al. did not evaluate the individual effects of the maternally and paternally inherited HLA alleles on odor perception. It appears that a preference for mates with intermediate levels of MHC matching is the optimal evolutionary strategy to preserve immunocompetence of offspring and that mating aims at achieving optimal genetic mixes rather than maximal mixes (Penn and Potts, 1999). The optimal genetic mix would vary depending on environmental pressures; for populations colonizing new ecosystems, a larger genetic mixing would be more beneficial in fighting against newly encountered pathogens compared to populations adapted to stable niches, for which keeping MHC alleles already selected through co-evolution with pathogens at a high frequency would be a better strategy (Ochoa and Jaffe, 1999).

The detection of MHC-mediated body odors may be the result of the close linkage between the MHC loci and polymorphic olfactory receptor genes, found in both humans

and mice (Fan et al., 1996; Ehlers et al., 2000; Younger et al., 2001). Candidates for MHC-specific odors in humans are soluble forms of the HLA proteins detected in urine, saliva and sweat (Wobst et al., 1999; Aultman et al., 1999), that may act as selective ligands for odor molecules, and by-products of MHC-specific bacteria colonization in skin or axillae (Pearse-Pratt et al., 1999). In mice, this role could be played by soluble MHC class I proteins circulating in serum that can bind specific volatile molecules for excretion in the urine (Singer et al., 1997).

Familial imprinting of MHC-specific odors is another mechanism to increase heterozygosity in offspring and to avoid inbreeding. A study done in mice showed that they prefer the odor of urine from an unrelated strain and avoid mice of the same strain as their mothers. This 'parent-of origin' effect, which occurs in mice of both sexes and might be thus due to autosomal imprinting, ensures inbreeding avoidance by diminishing preference for maternal urine and encouraging dispersal (Isles et al., 2001).

While odor cues seem to be essential for MHC-mediated mate choice and kin recognition in humans and rodents, they have been almost entirely ignored in avian studies because birds are generally thought of as having an unimportant or little used olfactory system (Zelano and Edwards, 2002). However, increasing number of studies showed that birds use odor cues in locating food (Nevitt, 2000) and in navigation and homing (Walraff, 2001). Kin recognition studies in birds, which did not rule out the role for olfaction, showed that individuals roosting together share higher DNA-fingerprinting band similarity than do individuals among roosts (Galeotti et al., 1997). Although the study of the relevance of MHC genes to avian mating and social systems is only at the beginning, and some biological processes influenced by MHC in mammals are

physiologically implausible for birds, it has been proposed that MHC, through its influence on health and vigor of individuals might affect mate choice (Zelano and Edwards, 2002).

Selective fertilization and abortion are reproductive mechanisms that favor MHC heterozygosity after mate selection. Fecundity studies performed in a Hutterite population revealed longer intervals to a detectable pregnancy and increased fetal loss rates in couples sharing HLA-DR and HLA-B alleles, respectively (Ober et al., 1992), which suggests a strong negative selection against HLA homozygotes. Deficits of homozygotes for HLA-A, -B, -C and HLA-DR and -DQ loci observed among the Hutterites showed that negative selection does not operate at the level of a single allele or locus (Kostyu et al., 1993). A large deficiency of homozygotes compared with Mendelian expectations was also observed in South Amerindian tribes (Black and Hedrick, 1997). Since this deficiency did not correlate with the age of the individuals sampled, as expected if there were increased relative mortality of the homozygotes due to increased exposure to infectious diseases with age, it was suggested that the selection might occur at the level of maternal-fetal interactions. Unsuccessful in vitro fertilization attempts, after recurrent spontaneous abortions, in couples sharing alleles at the HLA-B, HLA-DR and HLA-DQ loci also supports the negative selection against HLA homozygotes and the importance of maternal-fetal interactions (Ho et al., 1994).

It has been speculated that genetic compatibility at the MHC in birds could select against embryos before hatching, similar to the selective abortion in humans (Zelano and Edwards, 2002), based on evidence from congenic chicken lines that displayed different hatchability among MHC haplotypes (Abplanalp et al., 1992).

Therefore, interactions with pathogens and reproductive mechanisms act in concert to attain a level of MHC diversity that would allow a population to counteract the constant attacks of infectious agents in its environment. The selective forces that drive the MHC polymorphism do not aim at achieving the maximum level of diversity, as shown by the fact that not all possible combinations of MHC alleles occur and that certain allelic combinations have a higher frequency than the theoretical calculations predict. The difference between the frequency observed for a particular combination of alleles and that expected from the frequencies of the single alleles is known as linkage disequilibrium. Certain combinations of alleles, which provide resistance to pathogens would be favored and thus over-represented, whereas combinations that associate with harmful effects such as susceptibility to autoimmune disorders would have a lower frequency than predicted. In addition to natural selection, the recombination differences, especially the absence of recombination, in different haplotypes may favor certain allelic combinations. Recombination hotspots are not randomly distributed throughout the MHC, in both humans and mice, and this might dictate the frequency of allelic association. Another possibility, applicable at least to the human population, is that sufficient time or sufficient numbers of generations have not elapsed to allow for a combinatorial equilibrium among the alleles present in the founders of the population (reviewed by Beck and Trowsdale, 2000).

1.1.6 Viral interference with the expression of MHC class I molecules

The important role of the MHC class I antigen presentation pathway in the detection of virally infected cells by CTLs has been exploited by viruses in order to establish latent or chronic infections. Epitope mutation, a viral strategy taking advantage

of the structural constraints that limit the number of peptides that can be bound by each MHC class I molecule, has been documented for human immunodeficiency virus (HIV) (Wodarz and Nowak, 1999), Epstein-Barr virus (EBV) (De Campos-Lima et al., 1993) and hepatitis C virus (HCV) (Wang and Eckels, 1999). However, epitope variation is constrained by the necessity of retaining viral protein function and, consequently, evasion mechanisms that target the gene expression, assembly and surface display of MHC class I molecules, which are more general processes and less dependent on allelic variation, have evolved (Brodsky et al., 1999).

MHC class I gene expression is down regulated in tumorigenic adenovirus type 12 (Ad12)-transformed cell lines through strong DNA-binding activity of the repressor COUP-TFII to the MHC class I enhancer (Smirnov et al., 2001). The chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), which is up regulated in these transformed cells, can completely repress both non-activated and NF-κB activated MHC class I transcription (Smirnov et al., 2001; Hou et al., 2002). Cytokine induction of NF-κB has been shown to be able to stimulate class I gene expression only after COUP-TFII repression was relieved, which suggests that Ad12 has developed a mechanism of keeping the transcription of class I genes tightly repressed under various physiological conditions (Hou et al., 2002).

Two human cytomegalovirus (HCMV) proteins, US2 and US11, interfere with the MHC class I assembly immediately after glycosylation and translocation of the heavy chain (HC) into the ER. These proteins mediate reverse translocation of the HC from the ER through the Sec61 complex to the cytosol, where the HC is deglycosylated and degraded by the proteasome (Wiertz et al., 1996a; Wiertz et al, 1996b).

Retention of peptide-loaded MHC class I molecules in the ER is mediated by other two HCMV proteins, US3, which blocks the export of these molecules from the ER (Jones et al., 1996; Ahn et al., 1996), and US10, which retards but does not block the transport of class I molecules from the ER (Furman et al., 2002). E19, an adenovirus protein, prevents the transport of MHC class I molecules to the plasma membrane by forming stable complexes in the ER (Burgert and Kvist, 1985).

TAP-dependent transport of antigenic peptides to the ER is the target of the herpes simplex virus (HSV) ICP47 and the HCMV US6 proteins. ICP47, a small cytosolic protein, binds directly to the peptide-binding site in the TAP molecule but, unlike normal peptides, it is not translocated across the membrane and it remains associated with TAP preventing further peptide binding (Tomazin et al., 1996). US6, an ER membrane protein, inhibits ATP binding to TAP and thus deprives the transporter of its energy source (Hewitt et al., 2001). By interacting with the ER lumen-exposed loops of the TAP molecule, US6 induces a conformational change that inhibits both ATP binding and peptide translocation (Ahn et al., 1997).

Viral interference occurs even after MHC class I molecules reach the plasma membrane. Two Kaposi's sarcoma-associated herpesvirus (KSHV) proteins, K3 and K5, induce the rapid down-regulation of MHC class I molecules from the plasma membrane and further degradation by proteases in an acidic endocytic compartment (Coscoy and Ganem, 2000). Nef, an HIV-1 protein, mediates internalization of class I molecules, which, instead of being degraded, are further sequestered in the *trans*-Golgi network (Schwartz et al., 1996).

Viral interference with different steps in the antigen presentation pathway aims at inhibition of MHC class I surface expression and subsequent abrogation of CTL recognition. This strategy is counteracted by host NK cell-dependent immune response against cells with a reduced MHC class I surface expression. However, several viruses, such as HIV and HCMV, have developed mechanisms for avoiding an NK cell response. In the case of HIV, Nef causes internalization of HLA-A and HLA-B molecules but not HLA-C and HLA-E. The lower level of constitutive expression of HLA-C, compared with HLA-A and HLA-B, will less likely stimulate a vigorous CTL response and will still be able to prevent NK cell activation. Additionally, unaltered surface expression of HLA-E will further protect the infected cell from NK lysis (Cohen et al., 1999). In the case of HCMV, UL18, a virus specific protein that resembles the class I HC and binds host cell β_2-m (Browne et al., 1990), interacts with an inhibitory receptor on NK cells and therefore protects HCMV-infected cell from NK activitity (Cosman et al., 1999).

1.2 Organization of the MHC in Vertebrates

1.2.1 Mammals

Our current understanding of MHC genomic organization, phylogeny, gene functions and protein structure comes from two extensively studied mammalian systems: humans and mice. The clustering of the MHC genes in humans and rodents within a single genomic region, which gave this locus the designation of 'complex', has been attributed to selective pressure. Keeping this set of polymorphic genes as a unit may help avoid conflicts during positive and negative selection through the coordinate expression of these genes in different tissues and the coordinate T cell selection during ontogeny (Trowsdale, 1995). Linkage may also facilitate the exchange of sequences by non-

homologous recombination mechanisms such as gene conversion (Trowsdale, 2001). Additionally, clustering of immunomodulatory genes, such as TNF, and particular HLA alleles may ensure an optimum level of immune response even with such a large array of class I and class II molecules (Gruen and Weissman, 1997).

The canonical MHC arrangement into three areas: class I, class II and class III (Campbell and Trowsdale, 1993; Newell et al., 1994) seems to have been broadly conserved throughout mammals (Trowsdale, 1995). The genes within these three regions encode four functional categories: 1. antigen processing and presentation (class I and class II genes, LMPs, TAP and tapasin); 2. innate immunity, inflammation and regulation of immunity (class III genes such as C4, C2, Bf, cytokines and TNF); 3. intercellular interactions via MHC receptors and ligands; 4. functions unrelated to immunity (Kulski et al., 2002).

The human MHC genomic region covers 3.6 Mb of DNA on chromosome 6p21.3 with approximately 224 coding and non-coding genes (MHC Sequencing Consortium, 1999). Among the expressed loci, only 40% are involved to some degree in immunity (Trowsdale, 2001). MHC, also called the Human Leukocyte Antigen (HLA), represents one of the regions with the highest gene density in the human genome and it encodes the most polymorphic human proteins known to date, the class I and class II molecules (Trowsdale, 2001). The class I region includes the classical class I (class Ia) genes: HLA-A, -B and –C, the non-classical class I (class Ib) genes: HLA-E, -F and –G, MICA and MICB and other genes unrelated to the immune system (MHC Sequencing Consortium, 1999). Some of the non-HLA genes in the class I region encode proteins with functions in cell growth, DNA replication and repair and regulation of transcription (Shiina et al.,

1999a). The class II region contains the class II α and β genes, HLA-DR, -DP, -DQ, -DM and -DO and the genes involved in the class I antigen processing and presentation pathway, LMP2, LMP7, TAP1 and TAP2. HLA-DM and –DO do not function directly as presenting molecules, their role being to aid in peptide loading onto classical class II molecules. The so-called "extended class II region" includes the tapasin gene, the gene for RXRB, one transcription factor that regulates class I expression, and non-immune-related genes (MHC Sequencing Consortium, 1999). The class III region, located between the class I and class II regions, includes genes involved in the innate immunity and inflammation, such as the complement components C4 and factor B (Bf), the tumor necrosis factor (TNF) and HSP70 (MHC Sequencing Consortium, 1999). There are also non-expressed pseudogenes representing all three groups of sequences. Related genes, such as the β_2-microglobulin and the CD1 genes, are located on chromosome 15 and 1, respectively.

The mouse (*Mus musculus*) MHC, known as the H2 complex, is organized in many aspects like the human MHC, with the three distinct regions, class II, class III and class I extending in the same order, from centromere to telomere, on chromosome 17. The genes encoding the immunoproteasome subunits, LMP2 and LMP7, and the TAP1 and TAP2 proteins are localized in the class II region, same as in humans and the class II and class III regions contain similar number of genes but with smaller introns and smaller intergenic regions (Gasser et al., 1994). The class I region contains genes, pseudogenes and gene fragments that have been difficult to relate to human class I genes, which suggests independent genetic events, such as duplications, deletions and rearrangements through which they developed separately from the human orthologues (Trowsdale, 1995).

Two key differences between the human and the mouse MHC are the absence of MIC genes and the presence of additional classical class I gene clusters, centromeric of the class II region, in the mouse (Allcock et al., 1999).

Apart from the mouse, another rodent MHC extensively studied is that of the rat (*Rattus norvegicus*). Although the MHC genomic organization in the two species is similar, there are three striking differences. First, rat MHC class I-a genes are all located centromeric of the class II region, in a region designated RT1-A. No classical class I genes have been identified so far in the telomeric class I region, designated RT1-C/E/M (Dressel et al., 2001). Second, the number of MHC class I-a genes varies between haplotypes (Joly et al., 1996; Walter and Gunther, 2000). And third, rats have two major allelic forms of TAP2, with different specificities for the peptides that they transport into the ER (Howard, 1993). TAP heterodimers containing the TAP2A allele display a broad peptide specificity, like human TAP, while heterodimers containing the TAP2B allele show strong preference for peptides with hydrophobic C-terminal residues, like mouse TAP (Momburg et al., 1994b). This functional dimorphism accounts for the class I modification phenomenon (*cim*) in rats, a differential surface expression of the product of the RT1-A^a allele depending on the TAP2 allele present (Powis et al., 1991). The close linkage of the TAP, LMP and RT1-A genes may ensure coevolution of the apt combinations of alleles (Joly et al., 1994).

1.2.2 Birds

The chicken (*Gallus gallus)* MHC is the best characterized among avian and non-mammalian vertebrate MHCs. The reasons are due to the economic value of the poultry industry and to the availability of large outbred chicken populations that makes it

possible to study the effects of pathogens on populations with defined genetics on a scale unattainable with biomedical model species (Kaufman et al., 1999a). Initially described as a serological blood group, the chicken MHC, named the B locus, subsequently showed association with all of the functional phenomena characteristic of an MHC, such as rapid graft rejection and mixed lymphocyte reaction, graft-versus-host reaction and control of antigen-specific proliferation of lymphocytes (reviewed by Guillemot and Auffray, 1989). The B locus encodes the biochemical and functional equivalents of the mammalian MHC class I (B-F) and class II (B-L) molecules as well as a large family of immunogenic surface proteins (B-G), unique to birds (Kroemer et al., 1990a). The B-F and B-L proteins are highly polymorphic and display a tissue distribution, which mirrors that of the mammalian class I and class II, respectively. The B-G proteins are highly polymorphic as well and widely distributed, with predominant expression on erythrocytes (Kroemer et al., 1990a). These characteristics of the B-G proteins coupled with a strong immunogenicity and the genetic linkage to B-F/B-L account for the initial description of the B locus as a serological blood group.

The B locus is located on a microchromosome along with the nucleolar organizer region (NOR), which contains all of the chicken rRNA genes (Bloom and Bacon, 1985; Guillemot et al., 1988). The NOR separates the B locus from the restriction fragment pattern Y (Rfp-Y), a region that contains non-classical, class I-b, MHC genes (Afanassieff et al., 2001), with low polymorphism, low expression level and lack of strong effects on graft rejection, mixed lymphocyte reaction and disease resistance (Miller et al., 1994; Pharr et al., 1996; Vallejo et al., 1997). The B locus and the Rfp-Y,

which segregate independently (Miller et al., 1996), are probably the result of an *en bloc* duplication event (Kaufman et al., 1995).

The B-F/B-L region is simple and compact, with 19 genes present in 92 kb of DNA (Kaufman, 1999). The central portion of the region, between the class II (B-L) β genes and the class I (B-F) α genes, comprises 11 genes in 44 kb, with an average gene size of 4.4 kb, average intron size of 200 nucleotides and intergenic distances (excluding promoters) as short as 15 nucleotides, resulting in genes with one-third the size of their mammalian homologues (Kaufman, 1999). Moreover, this central region does not contain repetitive elements, pseudogenes or gene fragments, this compactness being consistent with the experimental evidence for little if any recombination in this region (Koch et al., 1983; Skjodt et al., 1985). By comparison, the C4 gene, the only gene of the class III region, located outside of the central portion, is approximately 18 kb, which is the same size as the human and murine homologues.

Other than a difference in size, between the chicken and the human and mouse MHC, there are also differences in the way this gene complex is organized. While orthologues of some mammalian genes are found in the B-F/B-L region, many are absent. There are classical class I (B-F) and TAP genes, but no LMP genes in the vicinity, unlike in mammals, where the TAP and LMP genes are intermingled. The lack of immunoproteasome elements LMP2, LMP7 and MECL1 might explain the unusual C termini (negatively charged) of peptides bound by some chicken classical class I alleles (Kaufman et al., 1995). Classical class II (B-L) β and DM α and DM β genes are present, but not classical class II α or DO genes (Kaufman, 1999). The single non-polymorphic classical class II α gene is located 5 cM away from the B locus (Kaufman et al., 1999a),

in contrast to the mammalian MHC, where the α/β gene pairs are closely linked. The class III region is represented only by the C4 gene, although the presence of more class III genes beyond the region that has been sequenced is not excluded (Kaufman et al., 1999a). There are genes in the B locus that would not be expected based on the MHC of mammals, such as the B-G gene, which is unique to birds and two genes that encode type II transmembrane proteins with apparent immunoreceptor tyrosine-based inhibitory motifs (ITIM) in the cytoplasmic tail (Kaufman et al., 1999a). One of them, transcribed in chicken NK cell lines but not in other haemopoietic cell types examined, encodes a product that closely resembles the natural killer C-type lectin-like receptor (NKLR), identifying it as the first non-mammalian NK receptor gene (Kaufman et al., 1999b). The order of the three regions in the chicken MHC differs from that in mammals, with the class III region being located outside the class I and class II. The fact that the highly polymorphic class I and class II genes are not separated by a huge class III region is another reason why the chicken MHC is so compact. One possibility is that the order found in the chicken MHC is primordial and the mammalian MHC arose by a rearrangement event that put the class III region between the class I and class II (Kaufman et al., 1999a). The organization of the genes in the chicken MHC regions also differs from mammals, with the TAP1 and TAP2 genes being flanked by the two class I genes, rather than being situated in the class II region. As well, the chicken tapasin gene is located between the two class II β genes, instead of centromeric to the class II β genes, as it is the case in humans and rodents (Kaufman et al., 1999a).

In most common chicken haplotypes, there is a single dominantly expressed class I molecule, with peptide binding specificity and expression level that can be used to

explain the striking associations of different haplotypes with resistance or susceptibility to certain infectious pathogens, such as Rous sarcoma virus and Marek's disease virus (MDV) (Kaufman, 1999). The gene encoding this dominantly expressed class I molecule, designated the major gene, has been shown to be transcribed at least tenfold more than the other class I gene (the minor gene), with no minor cDNA found in some haplotypes (Kaufman et al., 1995). Genomic analysis revealed that in all haplotypes examined the two class I genes flank the TAP genes with opposite transcriptional orientation, the class I promoters being located outside of the group (Kaufman et al., 1999a). The minor gene is located near the TAP1 gene and the major gene is adjacent to the TAP2 gene. While the proximal region of the promoters is virtually identical in the minor and major gene, the distal region, which includes the enhancer A element, is either altered in some haplotypes or deleted entirely in others, presumably leading to reduced transcription (Kaufman et al. 1999a). Additionaly, a disruption near the 5' end of the minor gene in some haplotypes is likely to be an explanation for the abolished expression (Kaufman et al., 1999a). The lower number of alleles of the minor gene compared with the major gene is consistent with the reduced expression of the minor gene, which associates with less selective pressure for diversification (Kaufman, 1999). Apparently, chickens invested in mechanisms to down regulate their minor gene so that it is not used very much, a detrimental strategy, given that having both genes expressed at the same level would increase the range of antigenic peptides presented and their chance to fight the pathogens (Kaufman et al., 1999a).

The single dominant expression of one class I gene in chicken has been attributed to the close proximity of the class I and TAP genes (Kaufman, 1999; Kaufman et al.,

1999a). These genes are only tens of nucleotides apart, which suggests that there is very little recombination between them and that they can co-evolve. Given that the major and minor class I molecules differ strongly in their peptide binding specificities and that there is also functional polymorphism in the TAP molecules, only the class I molecule with the same peptide binding specificity as the transporter will find peptides to bind and will be expressed on the surface. No matter the level of the other class I molecule, it will only bind the translocated peptides if it has the same peptide binding specificity. Therefore, the more restrictive the TAP, the more restrictive the tightly linked class I molecule and the less likely the peptide binding by a second class I molecule with a different specificity (Kaufman et al., 1999a). These closely linked genes co-evolve so that their products can interact and the longer the two alleles at adjacent genes stay together, the greater the chances that mutations in either or both alleles will confer a short term selective advantage to the individual carrying them (Kaufman, 1999). The resistance or susceptibility of a certain haplotype to infections with Rous sarcoma virus and Marek's disease virus (MDV) is therefore the consequence of the expression of a single class I molecule which can or cannot bind the peptide that confers protection (Kaufman et al., 1999a). By contrast, in mammals, the location of the TAP genes in the class II region force the TAP molecules to evolve to an "average best fit" for the peptides used by the products of the polymorphic class I genes, which switch frequently by recombination (Kaufman, 1999).

The simple and compact nature of the entire B-F/B-L region suggests a low level of recombination and co-evolution of genes in the chicken MHC as haplotypes, an idea supported by the fact that no recombinant has been found between the class I and class II

genes in over 6,000 progeny (Skjodt et al., 1985). The simplicity and reduced size of the chicken MHC, in comparison with mammals, led Kaufman et al. (1995) to suggest that it may contain the minimal number of genes essential for the function of an MHC and thus it may represent the "minimal essential MHC".

Another avian species for which the study of the organization of the MHC has been initiated is quail (*Coturnix japonica*). A preliminary comparative genome map between the quail and the chicken MHC shows a similar basic organization, with the TAP1 and TAP2 genes flanked by MHC class I loci, the class II region interspersed with tapasin, NKLR, and BG-like genes and no proteasome (LMP) genes in the vicinity (Shiina et al., 1999b; Kulski et al., 2002; Shiina et al., 2004). Like chicken, the class I region has an inverted duplication consisting of a class I gene linked to a TAP gene and is flanked by a class III gene, C4. The separation of MHC genes in two clusters, representing the B and Rfp-Y loci, has also been observed in quail. However, the quail MHC region is larger (180 kb) and less streamlined with more duplicated copies of the class I- and class II-like genes, NKLR and BG-like genes. There are seven class I and ten class II β-chain loci compared to only two copies of each in chicken (Shiina et al., 2004). In addition, the BG-like and NKLR genes, present in single copies in chicken, are more numerous in quail (Kulski, 2002; Shiina et al., 2004). Studies of the expression of the class I genes in quail identified four class I cDNA transcripts, which translate into proteins that are almost as homologous to the chicken class I B-F molecule as they are to each other (Shiina et al., 1999c). Two of the expressed class I genes are non-classical (Shiina et al., 2004), with genomic sequences that lack some introns (Shiina et al., 1999c) and the two corresponding loci not located in the current genomic map of the MHC

(Kulski et al, 2002). Structural comparison of the amino acid sequences of the four class I proteins with chicken and other known MHC class I proteins showed that the quail molecules have characteristic features required for functioning as antigen presenting glycoproteins (Shiina et al., 1999c). As well, the fact that the majority of amino acid differences between the four molecules clustered in the α1 and α2 domains implies that each one binds a different array of peptides with an isotype-specific motif. The higher number of expressed class I loci in the quail MHC might represent an adaptation to a large variety of pathogens encountered in the wild, compared with chicken, whose small number of expressed class I loci might reflect a bottleneck effect obtained through domestication (Shiina et al., 1999c). Alternatively, the quail MHC might have been generated in successive rounds of duplication within a simple MHC (Mesa et al., 2004). The presence of one duplicated module, containing MHC class II, B-G and lectin genes at one end and of another one, containing class I genes, at the other end (Kulski et al., 2002) are in favor of this hypothesis.

Studies of the genomic sequences and the expression of MHC class I genes in a passerine bird, the great reed warbler (*Acrocephalus arundinaceus*), revealed seven different genomic class I sequences in a single individual and eight cDNA transcripts, suggesting that there are at least four transcribed loci (Westerdahl et al., 1999). Of the seven genomic sequences, one had a deletion leading to a shift in the reading frame, indicating that it was not a functional gene. The amino acid sequences corresponding to the eight transcripts displayed features characteristic of functional MHC proteins and the amino acid differences were concentrated in the α1 and α2 domains indicating that the sequenced alleles are expressed and that there is a selection for diversity at the PBR

(Westerdahl, 1999). The number of class I loci and cDNA transcripts found so far suggest that the great reed warbler does not have a "minimal essential MHC" as it has been suggested for the chicken (Kaufman et al., 1995).

Sequence analysis of the MHC class II loci and transcripts in the great snipe (*Gallinago media*) (Ekblom et al., 2003), the red-winged blackbirds (*Agelaius phoeniceus*) (Gasper et al., 2001) and the Savannah sparrow (*Passerculus sanwichensis*) (Freeman-Galant et al., 2002) revealed the presence of higher numbers of loci and transcribed genes compared with chicken.

Although the genomic organization and the relative level of expression of the different MHC class I and class II loci has not been studied in other birds to the same extent as in chicken, preliminary data suggests that while the avian MHC remains simpler and smaller than that of mammals, various degrees of size and complexity can be found.

1.3 Hepatitis B virus (HBV)

1.3.1 Introduction

Human hepatitis B virus (HBV) is the prototype member of the family of hepadnaviridae, represented by hepatotropic, non-cytopathic DNA viruses. The other members of this family are the woodchuck hepatitis virus (WHV) (Summers et al., 1978), the ground squirrel hepatitis virus (GSHV) (Marion et al., 1980) and the duck hepatitis B virus (DHBV) (Mason et al., 1980). HBV infects only humans, which are a natural host, and chimpanzees, which can be infected experimentally.

Worldwide, HBV infection affects more than 2 billion people, among them approximately 350 million being chronic carriers of the virus (Lee, 1997). Although chronic carriers can remain asymptomatic for long periods, 15%-25% of them are

estimated to die prematurely from cirrhosis or hepatocellular carcinoma. The major modes of HBV transmission are vertical (perinatal infection of neonates born to HBV infected mothers), sexual and parenteral (through needles contaminated with bodily fluids from an infected individual) (Lin and Keeffe, 2001). While the infection in adults is usually transient, due to effective immune responses, more than 95% of the perinatally infected neonates become chronic carriers (Protzer and Schaller, 2000). The currently used vaccine can prevent HBV infection but it is ineffective as a therapeutic agent for chronic infection. Lamivudine and interferon-α2b, currently used in the treatment of chronic hepatitis B, can reduce progression to cirrhosis and hepatocellular carcinoma in some but not all of the cases and are incapable of achieving viral clearance and therefore of preventing a relapse (Lin and Keeffe, 2001).

1.3.2 Structure of HBV

The infectious HBV virion consists of a lipoprotein shell, formed by three envelope glycoproteins (S, M and L) and a lipid bilayer, and a nucleocapsid, which contains the viral DNA and the covalently attached viral polymerase (P) protein (Protzer and Schaller, 2000). The three envelope glycoproteins are thought to be involved in attachment of the virus to hepatocyte receptors and in subsequent internalization. The structural component of the nucleocapsid, the core (C) protein, was originally detected serologically as the hepatitis B core antigen (HBcAg). A non-structural, core-related peptide can be detected as serum HBeAg after its secretion from infected cells and serves as a marker of active viral replication. The viral genome is a partially double-stranded circular DNA of approximately 3.2 kb, which replicates through reverse transcription of an RNA pregenome (Protzer and Schaller, 2000). Upon entering the cell and uncoating,

the nucleocapsid delivers the partially double-stranded DNA genome to the nucleus, where host DNA repair enzymes complete and ligate the viral strands, yielding the covalently closed circular DNA (cccDNA). This serves as a template for transcription of a pregenomic and three classes of subgenomic RNAs. The pregenomic RNA acts as mRNA for the core (C) and polymerase (P) proteins, while the three subgenomic RNAs are translated into envelope proteins L, M and S and into X protein. Although its function remains poorly understood, *in vitro* assays showed that X protein has the ability to transactivate various viral and cellular promoters and suggested that it plays a major role in the initiation of hepatocellular transformation during an HBV infection (reviewed by Diao et al., 2001). Both woodchucks (Feitelson et al., 1993) and ducks (Chang et al., 2001) express X proteins with potential roles of promoting *in vivo* replication of WHV (Zoulim et al., 1994) and DHBV (Meier et al., 2003). The pregenomic RNA is co-packaged with the polymerase protein into the progeny capsids, where it is reverse transcribed into viral DNA. These progeny capsids can either reenter the nucleus to establish a pool of cccDNA, essential for the maintenance of infection, or be secreted as enveloped virions. Subviral particles, consisting mainly of S protein and no viral DNA, are also secreted (Protzer and Schaller, 2000). All nucleotides in the HBV genome have coding function in four overlapping open reading frames and the regulatory elements overlap with the coding regions. This extreme exploitation of genetic information makes most mutations disadvantageous for the virus and explains the relatively high level of sequence conservation among different HBV isolates (Protzer and Schaller, 2000).

1.3.3 Virus-host interaction in HBV infection

HBV is a non-cytopathic virus, the cause of liver injury being the host's immune response, mediated by CTLs that recognize HBcAg, presented on the surface of the hepatocytes. CTLs are the main effector arm of the anti-HBV immune response, with either protective or damaging effects, depending on their microenvironment and functional capacity (Bertoletti and Maini, 2000). A favorable balance between the levels of the virus and the virus-specific CTL recruitment is thought to result in the control of infection without histological signs of inflammation, whereas an inadequate CTL response might initiate massive infiltration of non-virus-specific mononuclear cells associated with liver inflammation (Bertoletti and Maini, 2000). Recognition by $CD8^+$T cells of HBV peptide fragments presented on the surface of the hepatocytes by MHC class I molecules leads to apoptosis, mediated by perforin or Fas ligand (Kondo et al., 1997), or to cytokine-dependent inactivation of the virus, mediated by IFNγ and TNFα (Guidotti et al., 1996). While the first mechanism results in lysis of hepatocytes and subsequent liver damage, the cytokine-mediated pathway allows for the selective degradation of replicating viral genomes and destabilization of viral RNA without the need to kill the infected cells (Guidotti et al., 1996). The virus specific $CD8^+$T cells are supported in their effector function at the site of infection by the $CD4^+$T helper cells that recognize viral peptides presented by MHC class II molecules on the surface of local macrophages. The activated $CD4^+$T helper cells secrete cytokines, which sustain CTL proliferation, increase the surface expression of MHC class I molecules on hepatocytes and decrease viral replication. In addition, they help with the priming of B cells, which, upon clonal expansion and differentiation into plasma cells, produce antibodies that

contribute to the clearance of circulating viral particles (Lee, 1997). NK and NKT cells, through the secretion of IFNγ and IFNα/β, also offer additional support in controlling the viral replication (Kakimi et al., 2000).

Although the factors that determine the outcome after acute HBV infection are poorly understood, it is believed that variations in host immune response rather than viral determinants are the critical variables (Chisari and Ferrari, 1995). Acute infection is characterized by a strong, polyclonal $CD4^+$ and $CD8^+$T cell response against HBV, which in 90%-95% of the cases results in viral clearance and immunity to reinfection, conferred by anti HBsAg antibodies. Chronic carrier state is characterized by a relatively weak and more narrowly focused T cell response (Chisari and Ferrari, 1995). In addition, chronic HBV infection is associated with a polarization of Th responses towards a Th2 type and a dysregulation of pro- and anti-inflamatory cytokines (Schlaak et al., 1999). Chronic stimulation of a relatively inefficient CTL response by persistent high levels of HBV results in sustained inflammatory and regenerative responses. These responses, which create the mutagenic and mitogenic stimuli for the development of DNA damage, are thought to favor the development of hepatocarcinoma (Nakamoto et al., 1998). Viral factors, such as vertical transmission, which associates with neonatal tolerance, infection of immunoprivileged sites, such as the hepatic tissue where immunological tolerance can be induced, antigen variation as a way to evade T cell recognition, may contribute to viral persistence in the context of an ineffective immune response (reviewed by Protzer and Schaller, 2000).

1.4 Natural Killer (NK) Cell Activating Receptors

1.4.1 Structure and functions

The NK cell activating receptors belong to two major families of molecules: the immunoglobulin superfamily (IgSF) and the C-type lectin superfamily (reviewed by Trowsdale et al., 2001). Receptors of the IgSF are encoded within the leukocyte Ig-like receptor complex (LRC), whereas the C-type lectin activating receptors are encoded within the natural killer complex (NKC) (Trowsdale et al., 2001).

The following section describes the general structure and functions of the activating receptors in the Ig superfamily, with emphasis on NKp46 and CHIR.

The Ig-like activating receptors have a variable number of C2-type Ig-like extracellular domains, a type I transmembrane region and a short cytoplasmic tail devoid of signaling elements. The presence of a positively charged residue (arginine or lysine) in their transmembrane portion allows binding to the negatively charged transmembrane aspartate residue of adaptor molecules and intracellular signaling (Martin et al., 2002). These adaptor proteins have extracellular domains that are too short to participate in ligand binding but contain cytoplasmic domains with immunoreceptor tyrosine-based activation motifs (ITAM), which allows association with downstream signaling molecules, such as ZAP70 and/or Syk-family tyrosine kinases (reviewed by Diefenbach and Raulet, 2001). Interaction between the activating receptors and their specific ligands, in the absence of the dominant inhibitory signal delivered by the MHC class I-specific inhibitory receptors, results in blastogenesis, cytokine production, cytotoxicity and migration of NK cells (Moretta et al., 2001).

1.4.1.1 NKp46

NKp46 is thought to be the main activating receptor for human NK cells and the only one conserved in humans, mice and rats (Bottino et al., 2000). NKp46 is a 46 kD type I transmembrane glycoprotein, 304 amino acids in length, belonging to the immunoglobulin superfamily (IgSF) (Pessino et al., 1998). The extracellular region consists of two cysteine-bridged C2-type Ig-like domains, followed by a stretch of amino acids, possibly forming a stem that connects the ectodomain with the transmembrane region. The transmembrane region contains a positively charged amino acid (Arg), which is thought to be involved in the association with signal-transducing molecules. The cytoplasmic portion is very short and does not contain ITAMs. Coupling to the intracytoplasmic transduction machinery is done by association with CD3ζ and FcεRIγ adaptor proteins, which have negatively charged residues in their transmembrane domain to interact with the Arg residue and cytoplasmic tails containing three and one ITAM, respectively (Moretta et al., 2001). The NKp46 molecule displays both N- and O-glycosylation sites, indicated by computer search and confirmed by treatment with N- and O-glycosidase (Pessino et al., 1998). The NKp46 gene is weakly expressed only in spleen and expression is confined to NK cells, which differentiates this molecule from the Ig-like transcript (ILT) molecules that generally have a broader cell distribution (Pessino et al., 1998). Analysis of the NKp46 cDNA sequences obtained from several unrelated donors showed that this gene does not display significant allelic variations between individuals (Pessino et al., 1998). Southern blot analysis revealed a relatively simple hybridization pattern implying the presence of a single gene or a few genes in the genome. Cross-hybridization, under low stringency conditions, with genomic DNA from

monkey, dog, rabbit, cow, mouse and rat suggests a significant cross-species conservation of the NKp46 gene (Pessino et al., 1998). The murine homologue of NKp46, known as mouse activating receptor (MAR-1), has already been cloned and characterized (Biassoni et al., 1999). Like NKp46, MAR-1 is a 46 kD type I transmembrane glycoprotein of the Ig superfamily, with two C2-type Ig-like domains, a transmembrane region containing a positively charged residue and a cytoplasmic tail lacking the immunoreceptor tyrosine-based activation motif (ITAM). The open reading frames (ORF) of MAR-1 and NKp46 cDNA sequences are 69% identical, whereas the two proteins share 58% identity (Biassoni et al., 1999). Like Nkp46, MAR-1 is selectively expressed by NK cells, irrespective of their state of activation. The rat homologue of NKp46, known as killer cell Ig-like receptor (KILR-1), has also been cloned and shown to display 59% identity at the amino acid level with the human receptor (Falco et al., 1999). NKp46 has been shown to recognize ligands such as haemagglutinin of influenza and haemagglutinin-neuraminidase of parainfluenza virus (Mandelboim et al., 2001).

1.4.1.2 CHIR

Chicken Ig-like receptors (CHIR)-A and -B are members of the paired Ig-like receptors (PIR) family, found in mice, and are currently recognized as the most distant PIR relatives characterized (Dennis et al., 2000). Receptors of the PIR family are expressed as activating and inhibitory isoforms with similar extracellular regions containing Ig-like domains that are coupled to distinctive transmembrane and cytoplasmic regions. The activating isoforms lack cytoplasmic signaling elements and they associate with transmembrane adaptor proteins that contain ITAMs, whereas the

inhibitory isoforms contain ITIMs in their cytoplasmic region (reviewed by Trowsdale et al., 2001; Takai and Ono, 2001). The chicken activating receptor candidate, CHIR-A, and the inhibitory counterpart, CHIR-B, are both type I transmembrane proteins with similar extracellular regions consisting of two Ig-like domains (Dennis et al., 2000). CHIR-A contains a positively charged histidine residue in its transmembrane region and a short cytoplasmic tail that lacks signaling elements. The positively charged transmembrane residue could promote association of CHIR-A with an ITAM-containing adaptor protein and thus allow it to function as an activating receptor. CHIR-B has an uncharged transmembrane region and a cytoplasmic tail with two ITIMs that identify it as an inhibitory receptor candidate. The two CHIR receptors share 74% amino acid identity in the extracellular region and show conservation of residues in areas likely to form β-stranded secondary structures, despite a low, 18%-26%, percentage similarity with the other members of the PIR family. Southern blot analysis revealed that CHIR-A and CHIR-B are members of a multigene family of Ig-like receptors. The coordinate expression of these two receptors on chicken T and B cells supposedly allows them to reciprocally modulate lymphocyte activation (Dennis et al., 2000).

1.4.2 Leucocyte Ig-like Receptor Complex (LRC)

The leucocyte Ig-like receptor complex (LRC), originally defined in humans, has been extended to mice based on orthologous genes present on syntenic chromosomes (Barten et al., 2001). This complex of loci is located on human chromosome 19 and mouse chromosome 7, and while in humans, it contains at least 40 members of the Ig superfamily genes, indicating a prolific duplication, in mice it is less complex (Trowsdale et al., 2001). The receptors encoded within the LRC can be classified into four groups,

based on the homology of their Ig-like domains and gene architecture (Martin et al., 2002). The first group includes the leukocyte Ig-like receptors (LILR) and the Ig-like transcripts (ILT) in humans and the paired Ig-like receptors (PIR) in mice. The second group of receptors comprises the killer-cell Ig-like receptors (KIR) and the platelet collagen receptor glycoprotein VI (GPVI), of which only GPVI has been identified in mice. The third group consists of the receptor for IgA Fc (FCAR) and NKp46 in humans and MAR-1/Ly94 in mice. The fourth group, present only in the human LRC, consists of the leukocyte-associated Ig-like receptors (LAIR).

The only true ortholog identified so far within the LRC is the NKp46 locus, conserved in humans, mice and rats, suggesting that primordial genes must have been in place before speciation between humans and mice (Martin et al., 2002). Gene structure similarities between the human LILRs and the mouse PIRs and the identification of chicken Ig-like receptors related to PIRs, the CHIRs, supports the existence of a common ancestor even before the divergence of the bird and mammalian lineages. The CHIR extracellular domains retain amino acid sequence motifs characteristic of the PIR family, despite an extensive sequence divergence. A hypothetical ancestral LRC, containing genes with some or all of the exons encoding the Ig-like domains found presently in the receptors, might have undergone intragenic duplication and shuffling of exons that led to the amplification and diversification of the receptors within the Ig-like families (Martin et al., 2002).

1.5 Rationale

The White Pekin ducks (*Anas platyrhynchos)* are natural hosts of two viruses of medical relevance, influenza A and hepatitis B. Influenza A infection in ducks is ubiquitous and, in most of the cases, does not associate with clinical signs of disease or detectable serum antibodies (Kida et al., 1980). The virus replicates in the intestinal epithelial cells of the duck, it is shed in high titers in their feces and, once in the lake water, can infect other individuals, since the excreted viral particles are still infectious after a week at 20°C, or after a month at 4°C (Webster et al., 1978). Multiple influenza strains can infect a duck and reassortant viruses arise through the interchange of individual genes between the strains. These newly derived strains can be passed to waterfowl and ultimately to man, thus making ducks the primordial reservoir of influenza A viruses for avian and mammalian species (Webster et al., 1992). There are multiple avian virus subtypes, only few of which have been found in nonavian hosts. This and the very low level of evolution observed for avian virus proteins compared with the rapid accumulation of mutations in proteins of avian subtypes in nonavian hosts suggest that influenza A viruses are a long-established pathogen in ducks and more transient in other hosts (Webster et al., 1992). The avirulent nature of influenza A infection in ducks, the result of many centuries of virus-host evolution, makes ducks a reservoir that ensures perpetuation of the virus and confers them a unique position in the history of influenza A viruses. Deadly outbreaks in human populations, such as the 1918, the 1957 and the 1968 pandemics, which killed millions of people, were caused by duck viruses, either after reassortment with the circulating human strain in swine or by direct transfer (Webster et al., 1992). The emergence of new human pandemic viruses, although rare, still occurs at

unpredictable intervals, on the order of decades, the most recent being the Asian bird flu, in January 2004. Understanding of influenza A infection in ducks could lead to farming of resistant ducks and thus to the elimination of the first step in human influenza outbreaks. Previous studies showed that ducks do not mount a characteristic secondary antibody response to influenza (Kida et al., 1980) and that, during their immune response, they make an IgY antibody that lacks the Fc region (Magor et al., 1992) and is therefore unable to interact with the antigen-presenting cells or activate the complement pathway. Due to this defective humoral immunity, ducks have to rely on cell-mediated immunity for the elimination of the virus.

Ducks are also a natural host of one of the few members of the hepadnaviridae family, the duck hepatitis B virus (DHBV), and a well-studied model for hepatitis B research. The limited host range of HBV, humans and chimpanzees, and the fact that it cannot be propagated in cultured cell lines are the factors that hamper the studies on the interaction between HBV and the immune system (Protzer and Schaller, 2000). HBV transgenic mice, in which acute hepatitis B is mimicked by an adoptive transfer of HBV specific CTLs, offer a model for the study of the HBV specific immune response (Chisari, 1996; Akbar and Onji, 1998). However, the absence of the viral cccDNA in the hepatocyte nucleus makes this model improper for testing the efficacy of therapeutic vaccines on cccDNA (Chisari, 1996). Woodchucks, which are naturally infected by a virus similar to HBV, represent an alternate model. Chronic carriers of the woodchuck hepatitis virus (WHV) have been used for preclinical evaluation of antiviral therapy for chronic HBV infection (Tennant and Gerin, 2001). The establishment of cccDNA in infected woodchuck hepatocytes is an advantage compared with the transgenic mouse

model, but the rapid progression to hepatocellular carcinoma represents a limitation for routine use (Tennant and Gerin, 2001). The duck hepatitis B virus (DHBV) shows structural features characteristic of the hepadnaviridae family, defined by the human HBV, and a similar preferential localization in the liver (Mason et al., 1980). Moreover, DHBV causes chronic or transient infection, depending on the age of the host at the time of viral acquisition, similar to HBV. Recently hatched ducks inoculated with DHBV develop chronic infection with minimal disease, while in older ducks inoculation with DHBV results in transient infection, with more extensive disease, the induction of neutralizing antibodies and immunity to re-infection (Jilbert et al., 1998). What makes the duck model even more appealing is the possibility to study the infection *in vitro*, in cultures of primary duck hepatocytes inoculated with DHBV obtained from congenitally infected ducks (Tuttleman et al., 1986). The ability of DHBV to undergo viral replication in cultured cells, a feature not shared with the human HBV, seriously reduces the time and costs of live animal research. Understanding the immune mechanisms that determine the outcome of the infection in ducks infected with DHBV could help develop effective therapies for chronic HBV infection in humans.

Our research aims at determining the characteristics of the duck MHC class I proteins that could influence the immune response to influenza A and hepatitis B viruses, such as diversity in the region contacting the antigen and modulation of expression in virally infected tissues. We are also investigating the possibility that ducks have a homologue of the human NKp46 or the chicken CHIR-A Ig-like activating receptors, conserved between species. Given its ability to recognize ligands such as haemagglutinin of influenza, NKp46 might be quite important in ducks.

1.6 Hypothesis and Objectives

At the beginning of this project, there was preliminary evidence suggesting that ducks dominantly express one MHC class I locus and that there is codominant expression of the alleles at this locus. The diversity of the MHC class I alleles in ducks was not known.

We hypothesized that: 1. Ducks have a limited allelic diversity at the dominantly expressed MHC class I locus. 2. MHC class I gene expression is down regulated during the duck hepatitis B virus (DHBV) replication. 3. Ducks have a homologue of the human NKp46 or the chicken CHIR-A Ig-like activating receptors.

To determine the level of allelic diversity at the dominantly expressed MHC class I locus, we cloned and sequenced both alleles expressed in six farmed ducks from three different colonies. We also assessed the MHC class I diversity in the genome, by amplifying MHC class I from genomic DNA of one of the ducks.

Examination of the MHC class I genes tissue expression revealed that there is variation between individuals. Therefore, to test the hypothesis of DHBV-induced down regulation of MHC class I gene expression, we cultured primary duck hepatocytes *in vitro*, infected them with DHBV and compared the transcriptional level between infected and non-infected cells from the same individual.

To investigate the presence of a duck homologue of NKp46, conserved among species, we performed genomic PCR amplification and Southern hybridization. The same homology-based approaches, plus RT-PCR and Northern hybridization, were taken to identify a homologue of the CHIR-A activating receptor in ducks.

2 METHODS

2.1 Laboratory ducks

White Pekin ducks were kindly provided by Dr. D. L. Tyrrell (University of Alberta) from the breeding stock of his duck colony. Animals were euthanized with Euthanyl and organs and blood were isolated. Organs were frozen immediately in liquid nitrogen and stored at –80°C. Blood samples were treated with heparin and kept at 4°C prior to isolation of DNA. Katharine Magor isolated the high molecular DNA.

2.2 Generation of the NKp46 probes

Degenerate and non-degenerate primers (Table 1.A) were designed based on the alignment of the exon 3, exon 4 and exon 7 sequences of human NKp46 (GenBank accession number AJ001383) and mouse MAR-1 (GenBank accession number AJ223765) genes. The forward primer HG46E3F1 and the reverse primer HG46E3R1 were used to amplify a 254 bp fragment from exon 3 (285 bp) of the human NKp46. The forward primer HG46E4F and the reverse primer HG46E4R were used to amplify a 257 bp fragment from exon 4 (279 bp) of the human NKp46. For each pair of primers, standard PCR conditions were used with an annealing temperature of 60°C. The products were gel purified using the QIAquick gel extraction Kit (Qiagen, Mississauga, ON) and sequenced to confirm their identity. They were used simultaneously as probes in Southern hybridizations of human, chicken and duck genomic DNA.

2.3 Generation of the CHIR probes

Degenerate and non-degenerate primers (Table 1.B) were designed based on the alignment of exon 2, exon 3 and exon 5 sequences of the chicken CHIR-A (AF306851), chicken CHIR-B (AF306852), mouse PIR-B (AF038149), rat PIR-B (AF16936), human

*Table 1: Oligonucleotides used for the amplification and sequencing of A. Human NKp46 and B. Chicken CHIR-A and CHIR-B products.

A. Human NKp46 primers

Primer name	Primer sequence	Length	Description
HG46E3F1	AACCGTTCATCTGGGCCGAGC	21	Exon 3 (Ig-like domain), forward
HG46E3R1	CAAGTTGCTGGGCTCTGAC	19	Exon 3 (Ig-like domain), reverse
HG46E3F2	GCCTTTTTGCCGTGGACAGACC	22	Exon 3 (Ig-like domain), forward
HG46E3R2	TGACCAGAGCTCCCCAACCCGATA	24	Exon 3 (Ig-like domain), reverse
HG46E4F	GTATGACACACCCACCCTCTCGG	23	Exon 4 (Ig-like domain), forward
HG46E4R	CTGGCTCACTGGGGAAAGACC	21	Exon 4 (Ig-like domain), reverse
HG46E7F	ATCACACTGCCCAGAATCTCCT	22	Exon 7 (Transmembrane domain), forward
HG46E7R	GCCTTCTCCTGCCTTCCCA	19	Exon 7 (Transmembrane domain), reverse
HG46E7FD	TGGGAYCAYACNGCNCARAA	20	Exon 7 (Transmembrane domain), forward, degenerate
HG46E7R1D	YCTYCTYCTNCCYTCCCA	18	Exon 7 (Transmembrane domain), reverse, degenerate
HG46E7R2D	NCGNCGNCGNCCYTCCCA	18	Exon 7 (Transmembrane domain), reverse, degenerate

*Table 1 continued

B. Chicken CHIR-A and CHIR-B primers

Primer name	Primer sequence	Length	Description
CHIRAD1F	CCCTCCCTGTTGCTGCACCCC	21	CHIR-A, Exon 2 (D1-Ig-like domain), forward
CHIRAD1RE	CAGCTTCACGGGGTCACTCTTCTC	24	CHIR-A, Exon 2 (D1-Ig-like domain), reverse
CHIRAD2F	CCCCCACCTGGCATTTCCCTGAGC	24	CHIR-A, Exon 3 (D2-Ig-like domain), forward
CHIRAD2RE	GGGTGAGAACAGAAGGCAGCA	21	CHIR-A, Exon 3 (D2-Ig-like domain), reverse
CHIRBD2F	CCCCCACCTGGCATTTCCCTC	21	CHIR-B, Exon 5 (ITIM domain), forward
CHIRB-ITIMRE	GTTGCAGCTGGGCGTAGGTCA	21	CHIR-B, Exon 5 (ITIM domain), reverse
CHIRA-DIF-D1	CARCARCTNCCNCGNCC	17	CHIR-A, Exon 2 (D1-Ig-like domain), forward, degenerate
CHIRA-D1F-D2	ACNGTNACNCTNCGNTG	17	CHIR-A, Exon 2 (D1-Ig-like domain), forward, degenerate
CHIRA-D2F-D	TAYCCNCCNCCNGAYATHTC	20	CHIR-A, Exon 3 (D2-Ig-like domain), forward, degenerate
CHIRA-D1R-D1	RTGYTARTGYCARTGYTA	18	CHIR-A, Exon 2 (D1-Ig-like domain), reverse, degenerate
CHIRA-D1R-D2	RTT NACNGGYTCYCTRTTRTC	21	CHIR-A, Exon 2 (D1-Ig-like domain), reverse, degenerate
CHIRA-D2R—D1	NGAYCARCTYTANGTNCC	18	CHIR-A, Exon 3 (D2-Ig-like domain), reverse, degenerate
CHIRA-D2R-D2	NCGYTANGAYCARCT YTA	18	CHIR-A, Exon 3 (D2-Ig-like domain), reverse, degenerate
CHIRB-ITIM-D	NAGRTGNAGRTGNGCYTA	18	CHIR-B, Exon 5 (ITIM domain), reverse, degenerate

LIR-1 (AF009220), human KIR2DL1 (AF022049), bovine Fcγ2R (2136749), human FcαR (U4774), human NKp46 (AJ001383), mouse NKp46 (AJ223765), rat NKp46 (AF082533) and mouse gp49-B (2997305) genes (Dennis et al., 2000). The forward primer CHIRAD1F and the reverse primer CHIRAD1RE were used to amplify a 261 bp fragment from exon 2 (279 bp) of the chicken CHIR-A gene. The forward primer CHIRAD2F and CHIRAD2RE were used to amplify a 252 bp fragment from exon 3 (294 bp) of the chicken CHIR-A gene. Standard PCR conditions, with an annealing temperature of 60°C, were used for each pair of primers. The products were gel purified using the QIAquick gel extraction Kit (Qiagen, Mississauga, ON) and sequenced to confirm their identity. They were used simultaneously as probes in Southern hybridizations of chicken and duck genomic DNA and in Northern hybridizations of various tissues of duck #64.

2.4 Southern hybridization

High molecular weight DNA from erythrocytes of humans, chickens and White Pekin ducks was digested to completion with 50 U of restriction enzymes (*Pst* I and *Pvu* II) for 6 hours at 37°C and then with another 50 U overnight, according to the manufacturer's protocol (New England Biolabs, Mississauga, ON). After digestion, the DNA was purified by phenol:chlorophorm extraction and precipitation with ethanol. The digested DNA (10 μg) was separated on 0.8% agarose gels in 0.5X TBE buffer for 22 hours at 30 V, blotted onto nylon transfer membranes (Nytran® SPC, Schleicher&Schuell Inc, Keene, N.H.) and immobilized by UV cross-linking (UV Stratalinker® 2400, Stratagene, La Jolla, CA). The blots were pre-hybridized at 42°C for 18 hours with 50% formamide, 5X Denhardt's reagent, 4X SSPE, 5% dextran sulfate,

1%SDS. Hybridization was done overnight with 100µg/ml salmon sperm DNA and specific probes. The probes were labeled with [α-32P]dCTP using the Prime-It® Random Primer Labeling Kit (Stratagene, La Jolla, CA). The blots were washed at low stringency [1X 100 ml: 1X SSPE and 0.1% SDS; 3X 300 ml: 1X SSPE and 0.1% SDS, at 52°C] and at high stringency [1X 100 ml: 1X SSPE and 0.1% SDS; 3X 300 ml: 0.1X SSPE; 0.1% SDS, at 65°C]. They were then exposed to Kodak X-OMAT AR scientific imaging film (Rochester, New York) at -80°C.

2.5 RT-PCR amplification of MHC class I alleles

First strand cDNA was made from 5 µg of spleen total RNA from duck #64, duck #95,duck #105, duck #132, duck #1 and duck #2, by priming with 0.5 µg of LINK-Tail (5'-TCTGAATTCTCGAGTCGACATCTTTTTTTTTTTTTTT-3') and extending with the Thermoscript RNase H⁻ Reverse Transcriptase kit, according to manufacturer's protocol (Invitrogen, Carlsbad, CA). The MHC class I forward primers D26 SP2 or D26 E2F1 and the reverse primer D26 3'UTR Rev (Table 2.A) were used for the amplification of MHC class I cDNA transcripts, using standard PCR conditions and an annealing temperature of 61°C. The products were gel purified using the QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON), ligated into the PCR® 2.1-TOPO® vector and transformed into TOP 10F' competent cells using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. At least two independent clones of each allele were sequenced, by primer walking, in both directions.

2.6 Amplification of genomic clones of MHC class I

Genomic MHC class I sequences from duck #64 were amplified from exon 2 (primer D26 E2F1 or D26 E2F2) to exon 6 (primer D26 CY1 Rev) or to 3'UTR (primer

Table 2.A: Oligonucleotides used for the amplification and sequencing of MHC class I cDNA and genomic clones. Primers were designed by Christine Mesa based on the alignment of four expressed MHC class I sequences from the cDNA library of duck 26 spleen.

Primer name	Primer sequence	Length	Description
D26 SP2	GGCTGCTGCTGGGGGTCCTG	20	Signal peptide primer that matches the four cDNA transcripts
D26 E2F1	GCCCCACTCCCTGCGCTA	18	Exon 2 (α1 domain) start, forward
D26 E2F2	CGGGAGCGCTACAACCAGAG	20	Exon 2 (α1 domain) end, forward
D26 E2R1	CTCTGGTTGTAGCGCTCCCG	20	Exon 2 (α1 domain) end, reverse
D26 PE3F1	ATGTATGGCTGTGACCTCCTC	21	Exon 3 (α2 domain), forward, last nucleotide mismatched in two cDNA transcripts
D26 E3F2	CCTGGAGAACACCTGCATTGAG	22	Exon 3 (α2 domain), forward
D26 PE3R1	GAGGAGGTCACAGCCATACAT	21	Exon 3 (α2 domain), reverse, first nucleotide mismatched in two cDNA transcripts
D26 E4F1	CCGCGACCCATCTCCATC	18	Exon 4 (α3 domain), forward
D26 E4R2	GTGAGCACGGCAGGACAAGGT	21	Exon 4 (α3 domain), reverse
D26 CY1 REV	TTGTAGCCCTTCCCCTTCTTC	21	Exon 6 (cytoplasmic 1, CY1, domain), reverse
D26 3'UTR Rev	GCAGATAGGAGATGTGAGAGGTTG	24	3'UTR, reverse

Table 2.B: Oligonucleotides used for the amplification of exon 4 of the duck #26 MHC class I gene. Primers were designed by Christine Mesa based on the alignment of the exon 4 sequences of chicken, quail and crane MHC class I genes.

Primer name	Primer sequence	Length	Description
CHE4F1	CCCGAGGTSCGAGTGTSG	18	Exon 4 (α3 domain), forward, degenerate
CHE4R1	CACGCGGCACYGGTACTTGTC	21	Exon 4 (α3 domain), reverse, degenerate

D26 3'UTR Rev) and from exon 3 (primer D26 E3F1 or D26 E3F2) to exon 6 (primer D26 CY1Rev) or to 3'UTR (primer D26 3'UTR Rev) (Table 2.A). Conditions for amplification of longer PCR products were used, the products were gel purified (QIAquick Gel Extraction Kit, Qiagen, Mississauga, ON) and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The longest clones (from exon 2 to 3'UTR) were completely sequenced.

2.7 DNA sequencing and analysis

Nucleotide sequences were determined on both strands with the cycle sequencing method, using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) on an automated ABI PRISM 377 DNA sequencer (PE Applied Biosystems Inc. Foster City, CA).

All chromatogram editing, nucleotide alignment, percent nucleotide identity analysis and primer design was performed using GeneTool (BioTools, Edmonton, Alberta) and by visual inspection. Protein alignment and percent aminoacid identity analysis was done using PepTool (BioTools, Edmonton, Alberta).

2.8 Generation of the MHC class I probe

Degenerate primers CHE4F1 and CHE4R1 (Table 2.B), previously designed by Christine Mesa based on the alignment of the exon 4 sequences of chicken, quail and crane MHC class I genes, were used to amplify a 228 bp fragment from the genomic DNA of duck #26. Standard PCR conditions were used, with an annealing temperature of 54°C. The product, consisting of several MHC class I sequences, was gel purified (QIAquick Gel Extraction Kit, Qiagen, Mississauga, ON) and used as a probe in Northern hybridizations.

2.9 Generation of the β-actin probe

A β-actin clone, 13B4, previously isolated and sequenced by Cynthia Radford from the cDNA library of duck #26 spleen, was used for insert recovery. A sample from this clone was plated and incubated overnight at 37°C. The pBK-CMV phagemid vector was isolated from individual colonies using a standard miniprep of plasmid DNA (Sambrook et al., 1989). The vector was digested overnight, at 37°C, with restriction enzymes (*Eco* RI and *Xho* I), the digested fragments run on a 0.8% agarose gel and the insert, purified (QIAquick Gel Extraction Kit, Qiagen, Mississauga, ON). The 3 kb insert showed 97% identity with the chicken β-actin gene over the 600 nt sequence, which included the start codon. This 3 kb fragment was used as a probe in Northern hybridizations.

2.10 Generation of the β_2-microglobulin probe

Kyle Thulien designed primers for the exon encoding the signal peptide and for the 3'UTR of the chicken β_2-microglobulin gene and used these primers to amplify the β_2-m genomic sequence from duck #26. The PCR product, cloned into the PCR® 2.1-TOPO® vector, was sequenced by Vidya Natarajan to confirm its identity. The 0.8 kb insert that she purified after *Eco* RI digestion showed 65% identity with the chicken β_2-microglobulin gene over the 400 nt sequence and was used as a probe in Northern hybridizations.

2.11 Generation of the DHBV probe

The DHBV probe was kindly provided by Dr. D. L. Tyrrell (University of Alberta). The pAlter-1 vector, after digestion overnight with *Eco* RI, liberated a 5.7 kb vector backbone and a 3 kb DHBV-16 monomer copy of the viral genome. The 3 Kb

insert was gel purified (QIAquick Gel Extraction Kit, Qiagen, Mississauga, ON) and used as a probe in Northern hybridizations.

2.12 Northern hybridization

Total RNA was isolated from the liver, kidney, lung, heart, spleen and intestine of duck #64, from the spleens of duck #95, duck #105, duck #129, duck #1 and duck #2 and from the cultured hepatocytes of duck #11 and duck #12, using TRIzol according to manufacturer's protocol (Gibco BRL, Burlington, ON). Cynthia Radford performed the isolation of total RNA from the spleen, heart, testes, kidney and liver of duck #132. The total RNA (10μg/sample) from different tissues was run on 1.2% agarose, 0.6% formaldehyde gels for 30 min at 60 V and then 2.5 hrs at 100 V. The gels were blotted onto nylon transfer membranes (Nytran® SPC, Schleicher&Schuell Inc, Keene, N.H.) and immobilized by UV cross-linking (UV Stratalinker® 2400, Stratagene, La Jolla, CA). The blots were pre-hybridized [50% formamide; 5X Denhardt's reagent; 4X SSPE; 1% SDS] for 4 hours at 42°C and hybridized for 22 hours at 42°C with 100μg/ml salmon sperm DNA and specific probes. The probes were labeled with [α-32P]dCTP using the Prime-It® Random Primer Labeling Kit (Stratagene, La Jolla, CA) and following the manufacturer's protocol. The blots were washed at either low or high stringency and exposed to Kodak X-OMAT AR scientific imaging film (Rochester, New York) at -80°C. The probes were stripped [3X300 ml: 0.1X SSPE and 0.5% SDS at 70°C], the membranes rinsed [1X200 ml: 2X SSPE at room temperature] and re-probed for 1-2 times.

2.13 *In vitro* culture and infection of primary duck hepatocytes with DHBV

Two DHBV-free ducks (#11 and #12), 7 day old, were euthanized with 0.5 ml Euthanyl, cleaned with detergent and their abdomen cut open to expose the liver and the

heart. A 24 GA catheter needle was inserted into the right ventricle and the portal vein was cut to drain the blood. The liver was first perfused with 200 ml MEM buffer until it turned pale and then with 100 ml collagenase, until well digested. After completely inhibiting the collagenase activity with EGTA and fetal calf serum at 4°C, the liver was cut out, put in a Petri dish with 30 ml of L-15 complete medium and gently teased until cells were well suspended. The suspension was filtered through sterile gauze and spun at 1000 rpm for 5 min. The pellet was washed 3 times with 30 ml of L-15 complete medium and resuspended in the same medium. The cells were counted and diluted with L-15 to a concentration of $16x10^6$ cells/100 mm dish/15 ml (duck #11) and of $21x10^6$ cells/100 mm dish/15 ml (duck #12). The cells were incubated overnight at 37°C, and the medium was changed the following day and every second day after, for 13 days. On day 3 post-plating, half of the plates from each duck were infected with DHBV, at a concentration of 100 genome particles/cell. Cells, both infected and non-infected, were harvested on day 3 and on day 7 post-infection, total RNA isolated, using TRIzol, and hybridized, in Northern blot assays, with MHC class I, β-actin and DHBV probes.

2.14 Polymerase chain reaction conditions

Amplifications were done using a GeneAmp® PCR System 9700 (PE Applied Biosystems Inc., Foster city, CA) based on the following standard conditions, with annealing temperatures specific for each primer pair used: 94°C for 5 min; 35 cycles at 95°C for 30 sec, (54°C to 62°C) for 30 sec, 72°C for 1 min 30 sec and 72°C for 3 min. Reactions were performed using 0.2 mM dNTP (Gibco BRL, Burlington, ON), 1X PCR buffer, 1X Solution Q, 2 units Taq polymerase (Qiagen, Mississauga, ON). Longer PCR products were amplified under the following conditions: 94°C for 1 min; 10 cycles of

94°C for 10 s, 60°C for 1 min, 68°C for 2 min; 25 cycles of 94°C for 10 s, 63°C for 1 min, 68°C for 2 min, with the extension time increasing by 2 s with every cycle; 68°C for 5 min. Human (150 ng), chicken (100 ng) and duck (100 ng) genomic DNA were used as templates with 20 pmol of each primer or 100 pmol, if the primer was degenerate.

3. RESULTS

3.1 NK cell activating receptors in ducks

3.1.1 Homology-based approaches to identify a duck homologue of NKp46

Our initial strategy to identify a duck homologue of NKp46 was amplification from genomic DNA using degenerate and non-degenerate primers (Table 1.A), based on conserved sequences between the human NKp46 and the mouse MAR-1. We used primer pairs for exons 3 and 4, which encode the two Ig-like domains, and exon 7, which encodes the transmembrane region. The different combinations of primers used amplified either multiple fragments, none of which were of the expected length, or no fragments at all. Sequencing of the amplified fragments and comparison with the sequences in GenBank showed no matches with relevant proteins in the database (data not shown).

A Southern blot of human, chicken and duck genomic DNA was hybridized with the two NKp46 probes, a 254 bp fragment from exon 3 and a 257 bp fragment from exon 4 of the human NKp46, simultaneously. Exon 3 and exon 4 shared 63% identity with the same exons of the murine MAR-1 gene and were the most conserved between the two genes (Biassoni et al., 1999). *Pst* I and *Pvu* II digested human, chicken and duck genomic DNA was blotted and hybridized with the two probes under low and high stringency conditions. Two hybridizing bands were visible in the human sample (in both the *Pst* I and the *Pvu* II digests) and no hybridizing bands were visible in the two chicken or the two duck samples (data not shown). The hybridization pattern seen was not affected by the level of stringency used.

3.1.2 Hybridization of duck genomic DNA with the CHIR probes

Chicken CHIR-A and CHIR-B are the most distant PIR relatives currently recognized, with the two Ig-like domains retaining characteristic amino acid sequence motifs of the PIR family, despite a relatively low level (25%) of sequence identity (Dennis et al., 2000). CHIR-A (AF306851) and CHIR-B (AF306852) share 85.7% nucleotide identity in exon 2 and exon 3, which encode the two Ig-like domains. To determine whether ducks have homologues of the chicken CHIRs, *Pst* I and *Pvu* II digested chicken and duck genomic DNA were hybridized simultaneously with the two CHIR probes, a 261 bp fragment from exon 2 and a 252 bp fragment from exon 3 of the CHIR-A gene (Figure 4). Hybridization of two chicken and four duck samples, under both low and high stringency conditions, showed multiple bands in the chicken and none in the duck samples, implying that the duck homologues, if present, share less than 70% similarity at the nucleotide level.

3.1.3 Hybridization of duck total RNA with the CHIR probes

Northern hybridization of total RNA from the liver, kidney, testes, heart and spleen of duck #132 and the spleen, kidney and intestine of duck #64 with the two CHIR probes, under low stringency conditions, showed a faint band of approximately 5 kb corresponding to the intestine sample (data not shown). Given the very low intensity of the signal compared with the background, we repeated the experiment to eliminate the possibility of a false positive result. When total RNA from the liver, spleen, lung, heart, kidney and intestine of duck #64 was hybridized simultaneously with the two CHIR probes, under the same conditions, only the bands corresponding to the 18S rRNA (1.8-2 kb) and 28S rRNA (4.6-5.3 kb) were visible, due to cross-hybridization (data not shown).

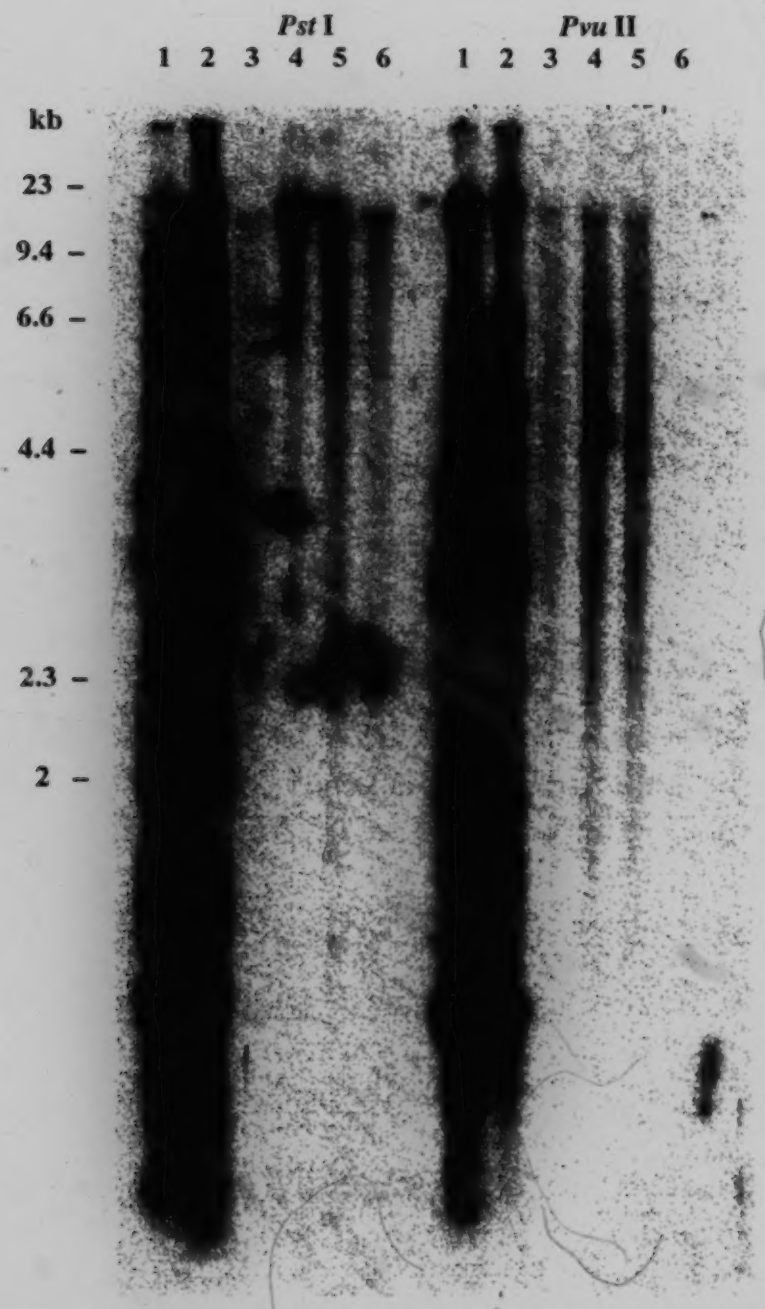

Figure 4: CHIR-A gene in chickens and ducks. *Pst* I and *Pvu* II digested DNA from chickens #1 and #2 (lanes 1-2) and from ducks #26, #95, #129, #132 (lanes 3-6) was hybridized with the CHIR probes under high stringency conditions. Sizes of HindIII-digested λ DNA are shown.

This result suggests that the similarity between the chicken CHIR-A gene and the duck homologue is below the limit of detection for Northern hybridization. Degenerate primers (Table 1.B) were also used in PCR amplification of the CHIR-A/B genes from duck #26 and #64 genomic DNA and in RT-PCR amplification using cDNA from duck #64 intestine. The different combinations of primers used did not yield any products.

3.2 Isolation of MHC class I cDNA and genomic clones

3.2.1 RT-PCR amplification of MHC class I alleles in six ducks

The MHC class I alleles expressed in six ducks were amplified by reverse-transcription PCR (RT-PCR). First strand cDNA was made from spleen total RNA coming from ducks #95, #105, #132, #64, #1 and #2. Ducks #95, #105 and #132 were from the same colony as duck #26, previously characterized (Mesa et al., 2004). Duck #64 was from outside the colony. These ducks were all DHBV-free. Ducks #1 and #2 were congenitally infected individuals from the Hutterite colony.

Amplification was performed using a forward primer (D26 SP2) in exon 1 (encodes the signal peptide) and a reverse primer (D26 3'UTR Rev) in the 3'UTR. The expressed class I sequences were further cloned in the TA overhang PCR 2.1 TOPO vector and the clones, sequenced in one direction with the forward primer D26 E2F1. Alignment of these sequences, which spanned the two exons that encode the α1- and α2-domain, allowed for the identification of the different MHC class I alleles expressed in each individual. Amplified products from two independent RT-PCR reactions were analyzed. For each unique sequence identified, two to five clones were sequenced completely on both strands by primer walking. In each individual, we identified two unique sequences, one of which was overwhelmingly amplified (Table 3).

Table 3: MHC class I alleles amplified from cDNA of six ducks. Numbers in brackets refer to the number of identical clones sequenced from each duck. *U*02* and *U*03* are the alleles identified by Christine Mesa in duck #26.

Duck No.	1(*U*02*)	2(*U*03*)	3	4	5	6
D95	D95.11 (26)	D95.2 (2)				
D105		D105.7 (42)				
D132	D132.5 (36)	D132.11 (3)				
D64			XD64.1 (33)	MD64.17 (3)		
D1					MD1.5 (32)	XD1.12 (2)
D2	MD2.16 (34)		XD2.24 (2)			

Ducks #95 and #132 shared the same pair of sequences, identical to *U*02* and *U*03*, previously identified in duck #26 as alleles at the same locus (Mesa et al., 2004) (Table 3). In duck #105 only one sequence, identical to *U*03*, was identified. The relative level of amplification of the *U*02* and *U*03* alleles was similar in ducks #95 (26/2) and #132 (36/3), with a bias toward *U*02*. Overwhelming representation of *U*02* was also observed in an unamplified cDNA library constructed from the spleen of duck #26, where, out of 31 clones isolated, 22 corresponded to *U*02* and four, to *U*03* (Mesa et al., 2004). Subsequent Northern hybridization of spleen and intestine mRNA from duck #26 with temperature-matched oligonucleotides specific for each of the two sequences, showed a higher expression of *U*02* in intestine (Mesa et al., 2004). Amplification of MHC class I sequences by RT-PCR, using spleen cDNA from duck #26 and D26 SP2, as a forward primer, and the oligo dT adaptor primer (Adap TTT), as a reverse primer, showed almost equal representation of the *U*02* (17 sequences) and the *U*03* (18 sequences) (Mesa et al., 2004). There was an exact match between the sequence of the reverse primer that we used, D26 3'UTR Rev, and the corresponding region of the *U*02* sequence, while there were three nucleotide differences between the primer sequence and the corresponding region of the *U*03* sequence. If this is also the case with all the pairs of unique sequences identified in the individuals studied, the bias observed is probably PCR-based and does not necessarily reflect the relative level of transcription.

Our RT-PCR amplification from six ducks identified two unique MHC class I sequences in each individual, bringing additional evidence for the dominant expression of one MHC class I gene in ducks and for the codominant expression of the class I alleles at a locus, previously reported in one individual (Mesa et al., 2004).

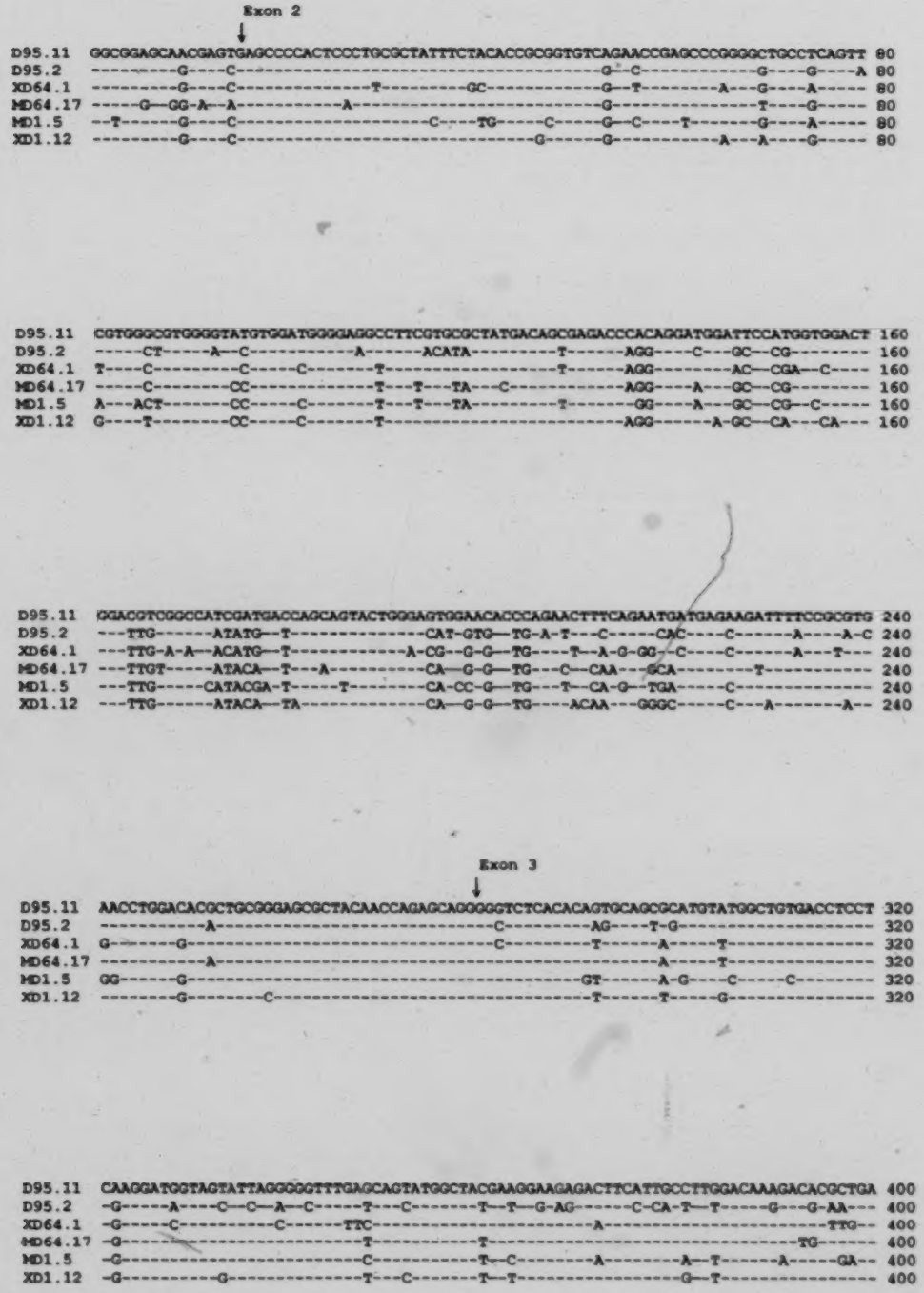

*Figure 5: Nucleotide alignment of the six duck MHC class I sequences amplified by RT-PCR (continued)

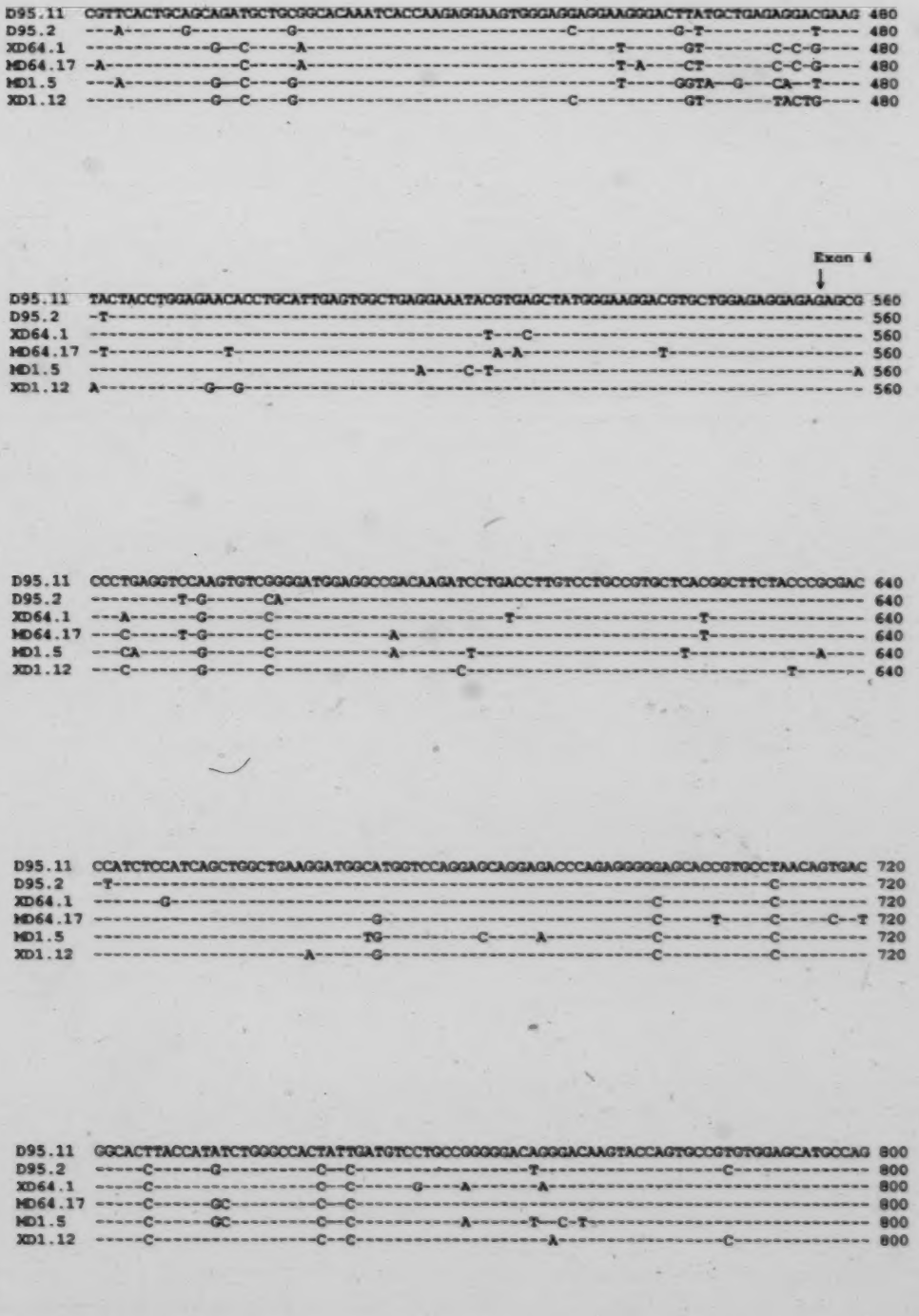

*Figure 5: Nucleotide alignment of the six duck MHC class I sequences amplified by RT-PCR (continued)

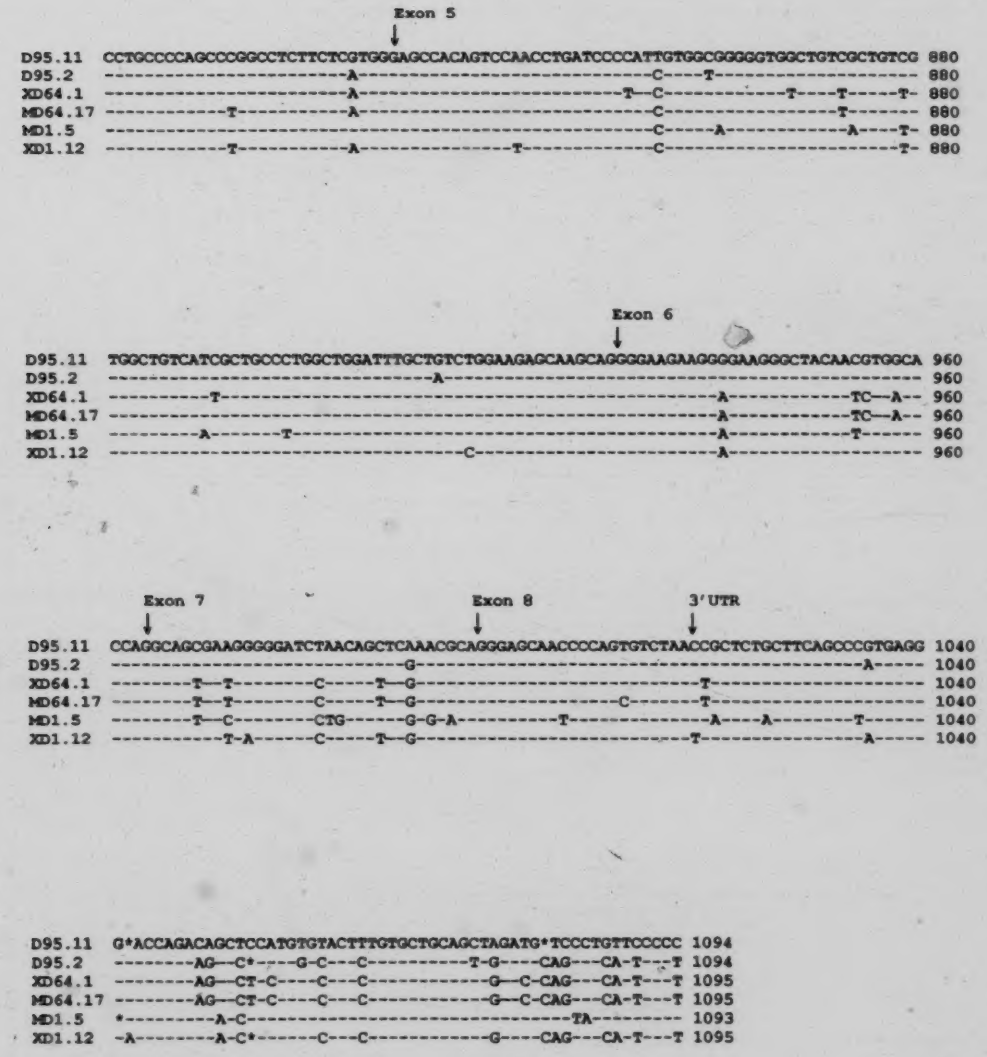

Figure 5: Nucleotide alignment of the six duck MHC class I sequences amplified by RT-PCR. Dashes (-) indicate identity with the sequence D95.11. Gaps, indicated by (*), are included to maximize the alignment. Exon boundaries are indicated.

Six different alleles were identified in the six individuals studied (Table 3; Figure 5). Each allele had an exon 2 (encodes the α1 domain) of 264 bp, an exon 3 (encodes the α2 domain) of 276 bp, an exon 4 (encodes the α3 domain) of 273 bp, an exon 5 (encodes the transmembrane domain) of 102 bp, an exon 6 (encodes the first cytoplasmic domain) of 33 bp, an exon 7 (encodes the second cytoplasmic domain) of 33 bp and an exon 8 (encodes the third cytoplasmic domain) of 21 bp (Figure 5). Additionally, each allelic sequence had part of exon 1 (encodes the signal peptide) and part of the 3'UTR, with a full length average of 1137 bp. The percent nucleotide identity of the entire allelic sequences ranged from 87% to 91.4% (primer sites not included), with the majority of the nucleotide substitutions clustered in exon 2 (77.7%-87.1% identity) and exon 3 (83%-92% identity). By comparison, exon 4 exhibited a percent nucleotide identity between 91.2% and 95.2%. There were two positions in exon 2 (70 and 81) and two positions in exon 3 (381 and 397) that displayed all four nucleotides (Figure 5). The majority of polymorphic positions displayed only two nucleotides.

3.2.2 Amplification of MHC class I genomic sequences from one duck

The genomic sequence of MHC class I was amplified in duck #64 from exon 2, using D26 E2F1 as a forward primer, to 3'UTR, using D26 3'UTR Rev as a reverse primer. The amplified product was cloned and, upon sequencing in one direction with the D26 E2F1 primer, four unique sequences were identified (Figure 6). For each genomic sequence identified, one clone was completely sequenced in both directions by primer walking. The exon-intron boundaries were determined based on comparison with the cDNA sequences amplified in duck #64. The exon-intron junctions were all type 1. One genomic sequence, D64g-2.8, matched the cDNA sequence XD64.1.

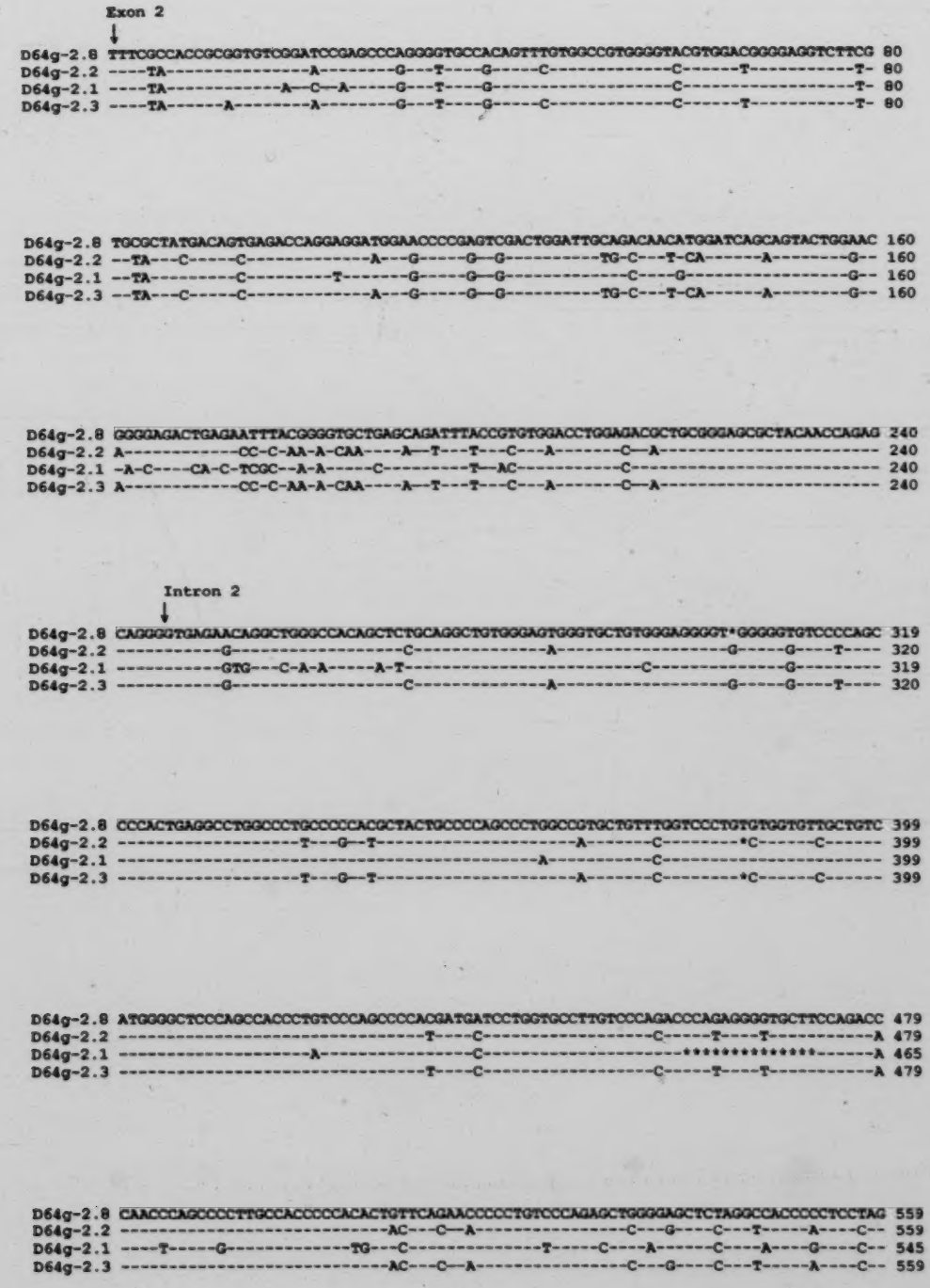

*Figure 6: Alignment of the four different MHC class I genomic sequences identified in duck #64 (continued)

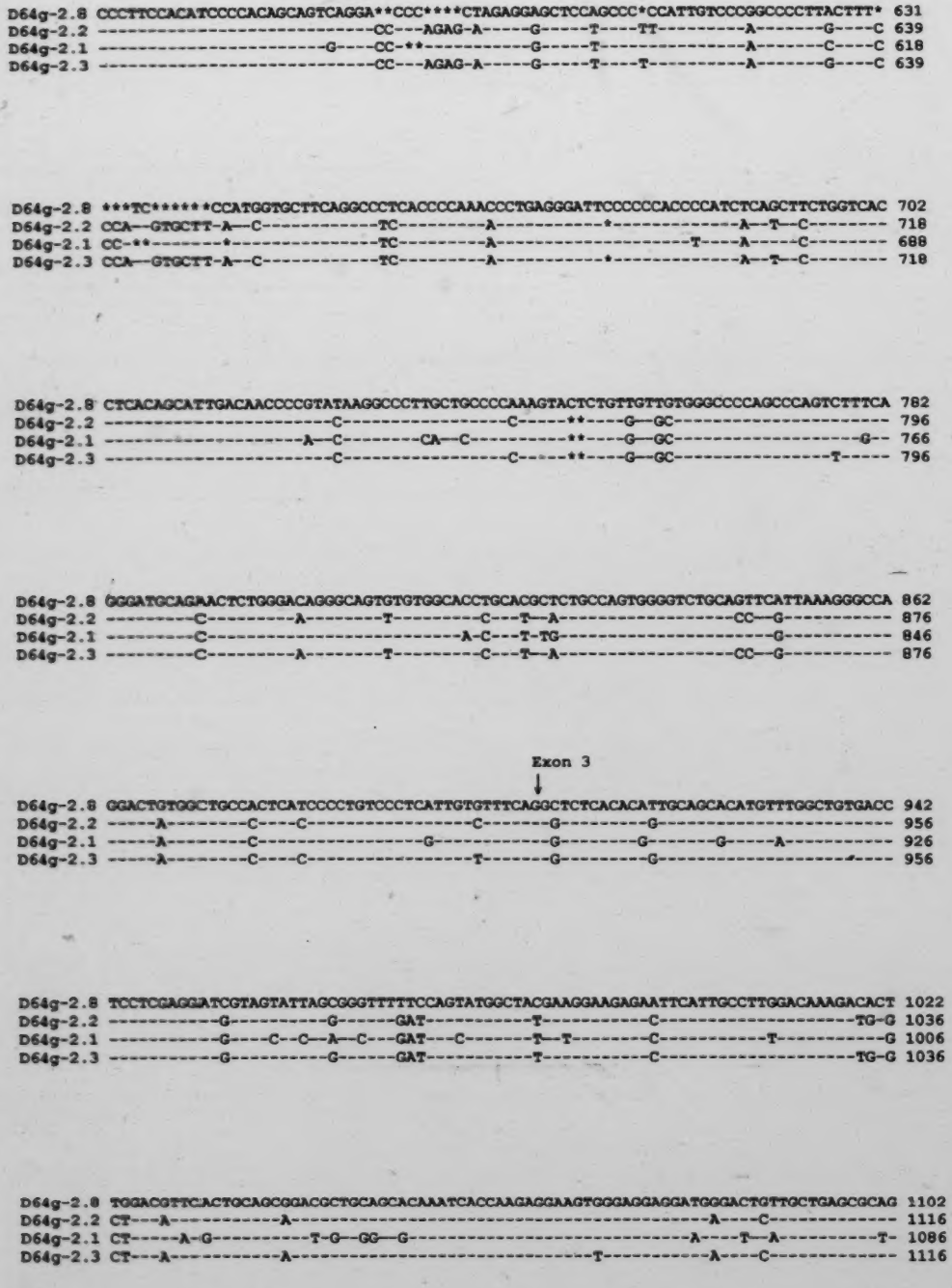

*Figure 6: Alignment of the four different MHC class I genomic sequences identified in duck #64 (continued)

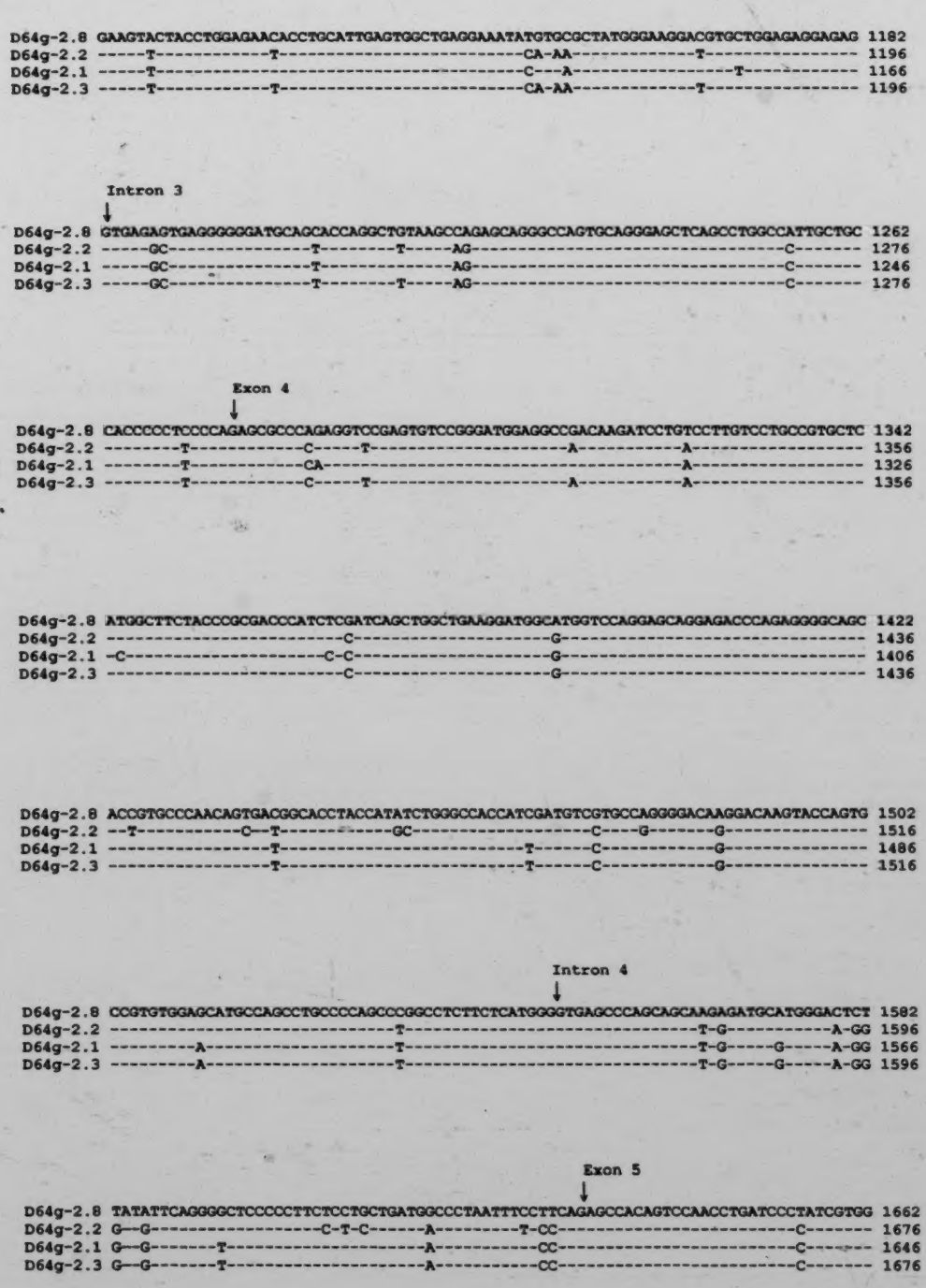

*Figure 6: Alignment of the four different MHC class I genomic sequences identified in duck #64 (continued)

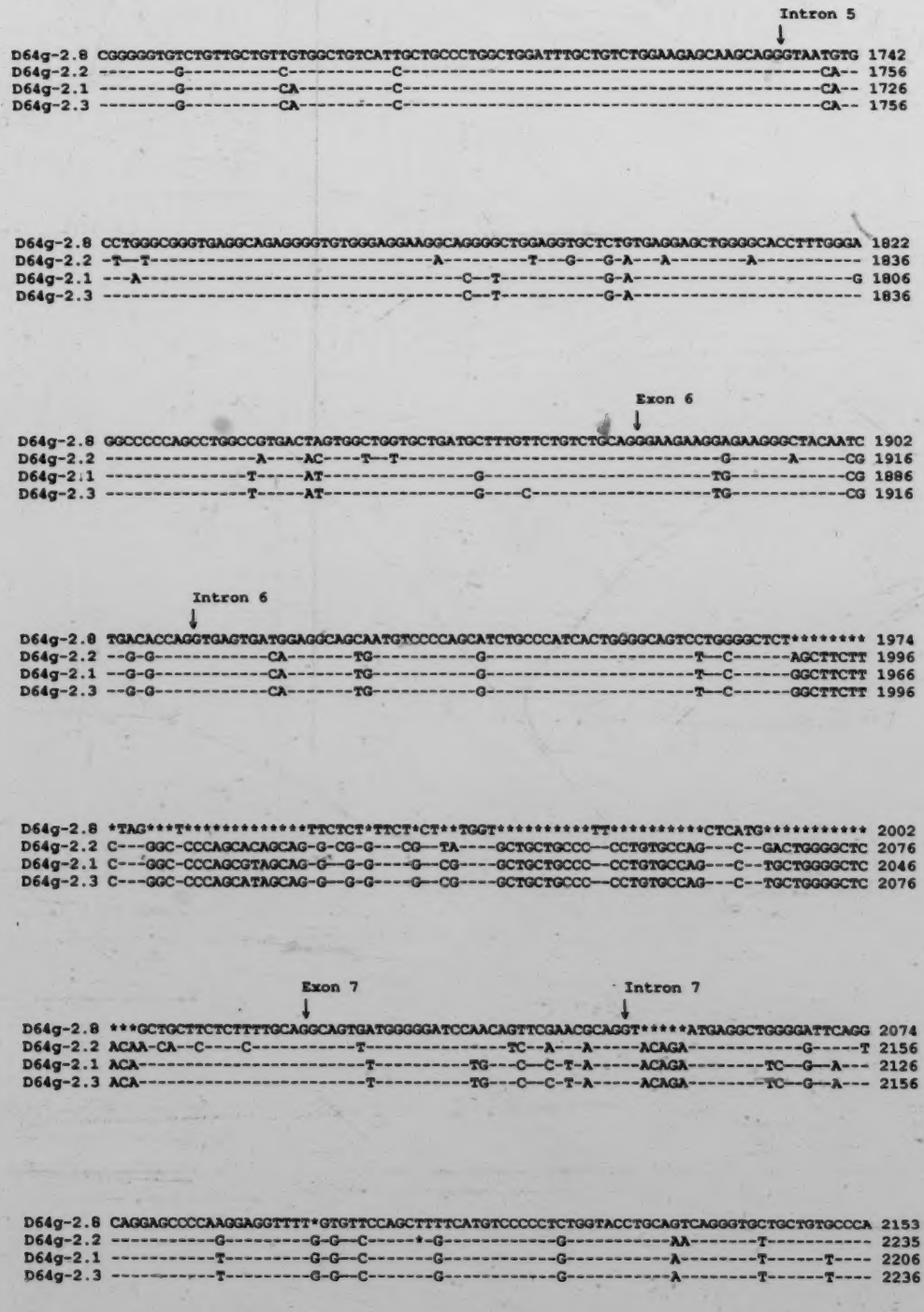

*Figure 6: Alignment of the four different MHC class I genomic sequences identified in duck #64 (continued)

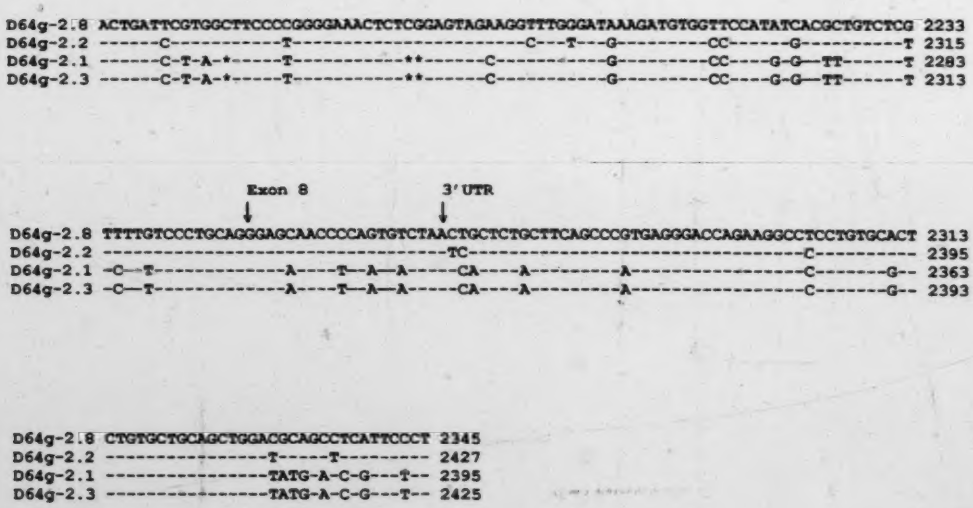

Figure 6: Alignment of the four different MHC class I genomic sequences identified in duck #64. Dashes indicate identity with the sequence D64g-2.8. Gaps, (*), are included to maximize the alignment. Exon-intron boundaries are indicated.

The four sequences varied in length, from 2,386 bp for D64g-2.8 to 2,468 bp for D64g-2.2. The percent nucleotide identity of the entire genomic sequences was between 88.4% and 96.2% (primer sites not included). Introns 3, 4 and 5 were of identical length, 94 bp, 83 bp and 144 bp, respectively. Introns 2, 6 and 7 displayed variation in length between the sequences. Intron 2 was 661 bp in D64g-2.8, 645 bp in D64g-2.1 and 675 bp in both D64g-2.2 and D64g-2.3. Intron 6 was 109 bp in D64g-2.8 and 172 bp in the other sequences. Intron 7 was 194 bp in D64g-2.8, 199 bp in D64g-2.2 and 197 bp in both D64g-2.1 and D64g-2.3. The size of the introns was similar to that of the chicken MHC class I introns (Kroemer et al., 1990b), with the exception of intron 2, which was twice the size of the corresponding chicken intron. All four sequences were translatable.

3.2.3 MHC class I amino acid sequences

The deduced amino acid sequences corresponding to the six different MHC class I alleles identified were aligned and compared (Figure 7). The domains were assigned based on comparison with the chicken (Hunt and Fulton, 1998), quail (Shiina et al., 1999c), great reed warbler (Westerdahl et al., 1999), woodchuck (Zhou et al., 2002) and the HLA-A2 (Koller and Orr, 1985) MHC class I protein sequences. Each sequence had an α1 domain of 88 amino acids, an α2 domain of 92 amino acids, an α3 domain of 91 amino acids, a transmembrane region of 34 amino acids and three cytoplasmic domains of 11, 11 and 6 amino acids. The percent amino acid identity of the entire sequences ranged from 79.6% to 87.7%, with the majority of the variable residues being located within the peptide binding region (PBR), formed by the α1 and α2 domains. The two domains displayed percent amino acid identities between 62.5% and 79.6%, for α1, and

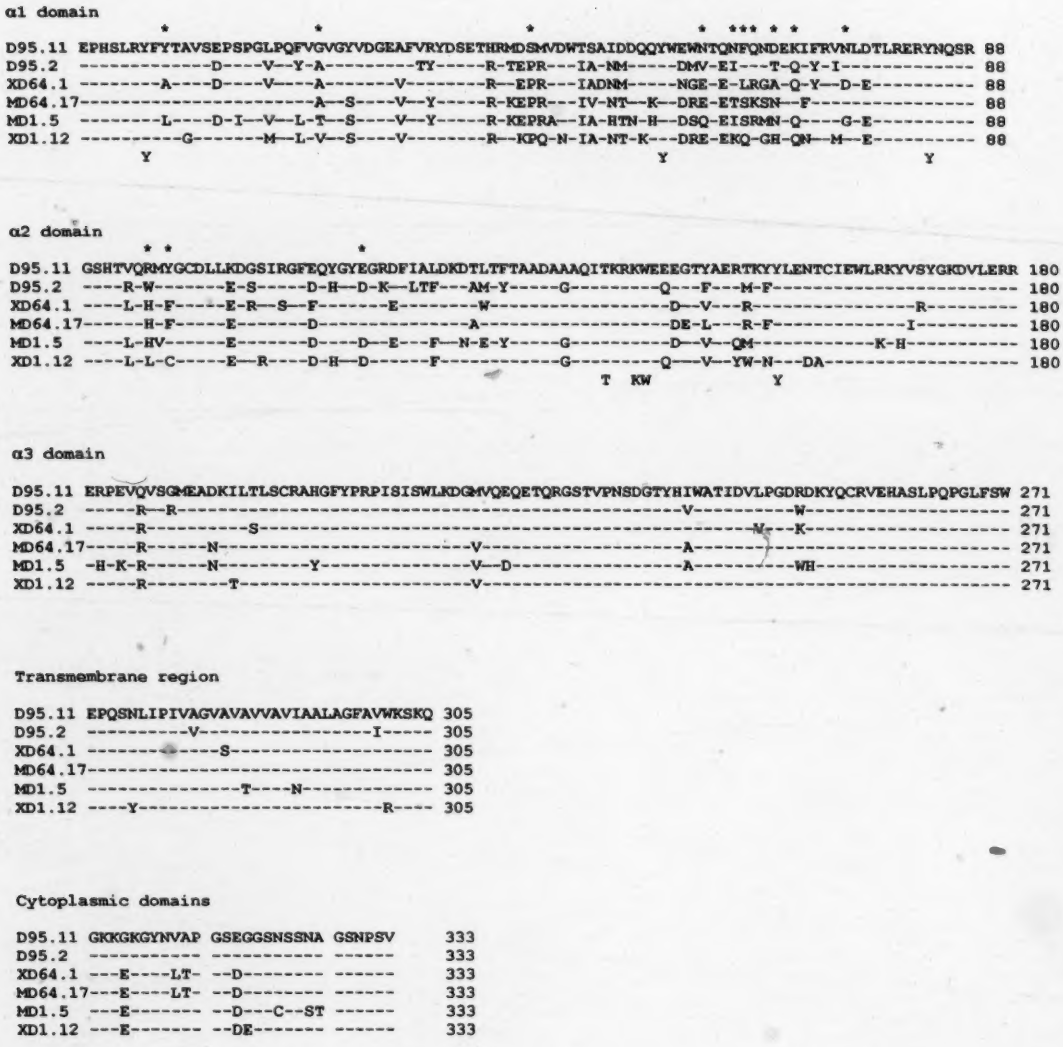

Figure 7: Alignment of the predicted amino acid sequence of the six MHC class I alleles identified in the six ducks. Dashes, (-), indicate identity with the sequence D95.11. Invariant amino acid residues at positions that anchor the peptide-terminal main chain atoms are indicated below the alignment. Positions of high diversity, conserved between human HLA and duck MHC class I sequences, are indicated by (*) above the alignment.

between 75% and 88%, for α2. A lower variability in the α2 domain in comparison with the α1 domain was also observed in the alignments of chicken (Hunt and Fulton, 1998; Livant et al., 2004) and quail (Shiina et al., 1999c) class I protein sequences. By comparison, the percent identities within the α3 domain were between 87.6% and 96.1%, suggesting selective pressure for polymorphism in the region contacting the antigen and for conservation in the region interacting with β_2-m and the CD8 co-receptor. There were 41/88 and 34/92 polymorphic positions within the α1 and α2 domain respectively and only 14/91, within the α3 domain. Many of the amino acid changes in the α1 and α2 domains were not conservative implying that these proteins have different peptide binding characteristics. In addition, many of the polymorphic positions in these two domains displayed at least three amino acids. By comparison, there were only two positions in the α3 domain exhibiting more than two residues.

Interestingly, a cluster of highly variable residues in the α1 domain, was found in a region similar to human (Parham et al., 1988), chicken (Hunt and Fulton, 1998; Livant et al., 2004), quail (Shiina et al., 1999c) and reed warbler (Westerdahl et al., 1999) MHC class I proteins. This region, between residues 60 and 80, is part of the α-helix of the α1-domain in the HLA-A2 molecule (Saper et al., 1991) and was predicted to be part of the same region of the α1-domain in the quail (Shiina et al., 1999c) and in the chicken (Kaufman et al., 1992). These predictions were made based on conservation of the characteristic features required for functioning as antigen presenting proteins, such as the positions of high amino acid variability, the intra- and inter-domain contact residues and the salt bridges, among the class I molecules from the three species. This cluster of polymorphic positions was larger in the duck, chicken (Hunt and Fulton, 1998; Livant et

al., 2004) and quail (Shiina et al., 1999c) class I sequences compared with the HLA sequences (Parham et al., 1988) and could be extended to 40 residues, between amino acids 40 and 80. There are eleven residues of high diversity (residues 62, 65, 66, 67, 69, 70, 71, 74, 76, 77 and 80) within this cluster in the HLA sequences (Parham et al., 1988). Seven of them were conserved in the duck sequences (62, 65, 66, 67, 69, 71 and 76). The position of a second cluster of variable residues, with a lower magnitude of variability though, between amino acids 145 and 160, appeared to be conserved between ducks, humans (Parham et al., 1988) and chickens (Hunt and Fulton, 1998; Livant et al., 2004). This cluster corresponds to the α-helix in the α2-domain in the human HLA-A2 (Saper et al., 1991) and to the predicted similar region in the chicken class I molecule (Kaufman et al., 1992). Not only the position but also the lower magnitude of variability of this cluster in the α-helix of the α2-domain was conserved in the duck, chicken and human sequences, suggesting selection for lower diversity in the α2-helices.

In humans, the β-strand region of the PBR displayed four positions of high variability (95, 97, 114 and 116) in the α2-domain and three positions (9, 24 and 45) in the α1-domain (Parham et al., 1988). Three of the four positions in the α2-domain (95, 97 and 116) and all three positions in the α1-domain were conserved in our duck sequences. By comparison, only positions 95 and 97 in the α2-domain and positions 9 and 24 were conserved in chicken (Hunt and Fulton, 1998; Livant et al., 2004) and quail (Shiina et al., 1999c).

In addition to conservation of the positions of high variability, the duck protein sequences showed conservation of the residues that are generally invariant in classical class I molecules. Seven of the nine residues that anchor the peptide amino- and carboxy-

terminal main-chain atoms (Y7, Y59, Y84, Y123, T143, K146, W147, Y159, Y171), generally conserved in the classical class I molecules throughout evolution (Kaufman et al., 1994; Shum et al., 1999) were found in our duck sequences. These were Y7, $Y58^{Y59}$, Y84, $T140^{T143}$, $K143^{K146}$, $W144^{W147}$, $Y157^{Y159}$. Position 123 in our aligned sequences displayed either L or F, which were seen at this position in other non-mammalian species (Shum et al., 1999). Other residues that are characteristic of class I molecules, the N-linked glycosylation site in the α1 domain (N86) and a phosphorylation site in the cytoplasmic domain (Y312) (Guild et al., 1983), were also conserved in our duck sequences ($N85^{86}$).

This data suggests that the duck proteins are classical MHC class I molecules with different peptide binding specificities.

3.3 Expression of MHC class I genes in ducks

3.3.1 Expression of MHC class I genes in duck tissues

To determine the level of expression of the MHC class I genes in various duck tissues, we performed Northern blot analysis. Two healthy individuals, duck #64 and duck #132, were selected. Total RNA isolated from the liver, kidney, lung, heart, spleen and intestine of duck #64 and from the heart, spleen, testes, kidney and liver of duck #132 was hybridized, under high stringency conditions, with the MHC class I probe (Figure 8 and Figure 9). This probe consisted of several MHC class I exon 4 sequences from duck #26. Hybridization with the β-actin probe showed different levels of expression between tissues and thus we could not use the β-actin probe as a control. Liver, spleen, lung and intestine showed significantly higher levels of expression, compared with heart, kidney and testes. In addition to the intense hybridizing band of

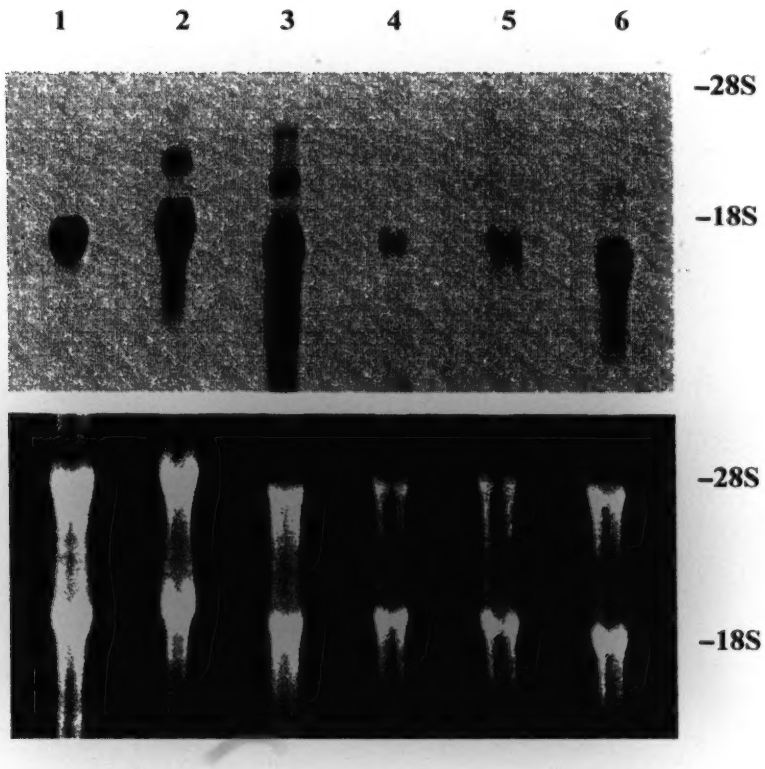

Figure 8: Northern blot analysis of MHC class I expression in different tissues of duck #64. Total RNA from various tissues was separated, transferred to a blot and hybridized with the MHC class I probe. Gel picture is shown for a comparison of the amount of RNA loaded in each lane. Positions of the 18S and 28S rRNA are labeled for reference. 1: liver; 2: spleen; 3: lung; 4: heart; 5: kidney; 6: intestine.

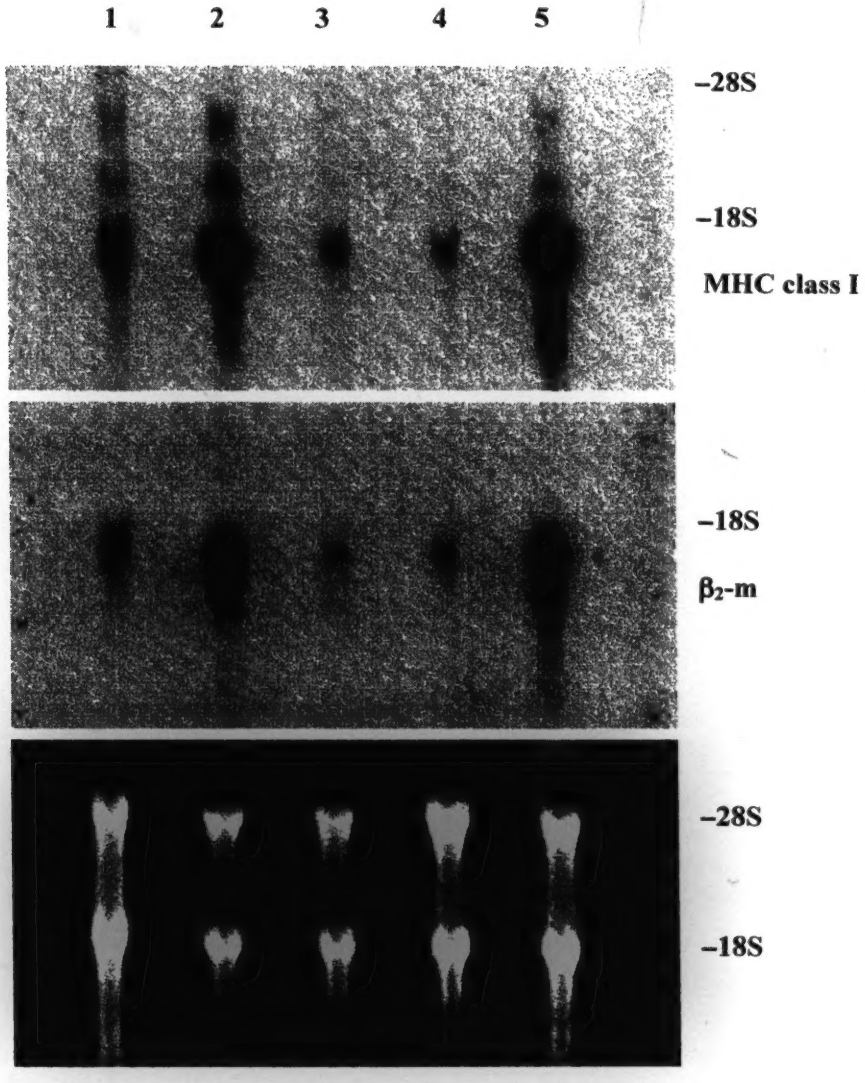

Figure 9: Northern blot analysis of MHC class I and β_2-microglobulin expression in different tissues of duck #132. Total RNA from various tissues was separated, transferred to a blot and hybridized with the MHC class I probe. After probe stripping, the blot was re-hybridized with the β_2-microglobulin probe. Gel picture is shown for a comparison of the amount of RNA loaded in each lane. Positions of 18S RNA and 28S RNA are labeled for reference.1: heart; 2: spleen; 3: testes; 4: kidney; 5: liver.

approximately 1.2 kb present in all samples, two more bands, approximately 3 kb and 4.5 kb, corresponding to unspliced pre-mRNA were faintly visible. These results suggest that there is significant transcription of MHC class I products 1.2 kb in length, which is in accordance with what we found through sequencing analysis. The cDNA clones that we sequenced from six ducks, using a forward primer in exon 1 (encodes the signal peptide) and a reverse primer in the 3'UTR were, on average, 1,137 bp in length. The results also suggest that there is a significantly high level of constitutive expression of the MHC class I genes in tissues that are targets for influenza A and hepatitis B infection.

3.3.2 Expression of β_2-m genes in duck tissues

The constitutive level of β_2-m gene expression was assessed by Northern blot in various tissues from one individual. Total RNA from the heart, spleen, testes, kidney and liver of duck #132 was hybridized, under high stringency conditions, with the β_2-m probe (Figure 9). The membrane had been previously hybridized with MHC class I and the probe stripped. One intense band of approximately 1 kb and another faint band of approximately 0.5 kb were visible. The length of the primary RNA transcript of the chicken β_2-m is 1038 b (Riegert et al., 1996), comparable to what we saw in duck #132. The presence of the 0.5 kb transcript is probably due to the existence of an alternate polyadenylation site. There were high levels of β_2-m gene expression in spleen and liver and low, in heart, testes and kidney, a pattern similar with the one observed for MHC class I genes, suggesting that the constitutive expression of the two genes is correlated. Studies performed in human cultured cells showed that the constitutive level of β_2-m protein is sufficient to support an increase of up to 75% in the heavy chain expression (Johnson and Mook-Kanamori, 2000). The increase in β_2-m required to support the

cytokine-induced levels of HLA class I expression in infected cells, which could be 10-fold higher than normal, is achieved by coordinate expression of class I HC and β_2-m induced by IFNs and TNF (Johnson and Pober, 1990).

3.3.3 Expression of MHC class I genes in different individuals

To determine if the level of MHC class I gene expression is the same between individuals, we hybridized total RNA from the spleens of five ducks, #95, #105, #129, #132 and #64, with the MHC class I probe, under high stringency conditions (Figure 10). After stripping the MHC class I probe, the membrane was re-hybridized with the β-actin probe, under the same conditions (Figure 10). The three hybridizing bands corresponding to the MHC class I 1.2 kb mRNA and 3 kb and 4.5 kb unspliced pre-mRNA were visible after the first round of hybridization. Two bands, one intense of approximately 3 kb and one very faint of approximately 1.5 kb, were visible after using the β-actin probe. The level of class I expression appeared to be slightly higher in duck #129 compared with duck #132 (at relatively similar signal intensities for MHC class I, the signal intensity for β-actin was significantly lower in duck #129). Similarly, the level of class I expression in duck #129 appeared to be slightly higher than in duck #64. As well, the difference in β-actin signal intensity between duck #95 and duck #105 was significantly higher than the difference in class I signal intensity, implying that duck #95 has a higher level of MHC class I expression than duck #105. Sequence analysis of the cDNA transcripts showed that duck #95 expresses the *U*02* and *U*03* alleles, whereas duck #105 is homozygous for *U*03*. This difference in the level of class I gene expression between individuals, present even when they are MHC class I matched, makes it thus difficult to test the hypothesis of virally induced down regulation of MHC class I gene expression. To avoid

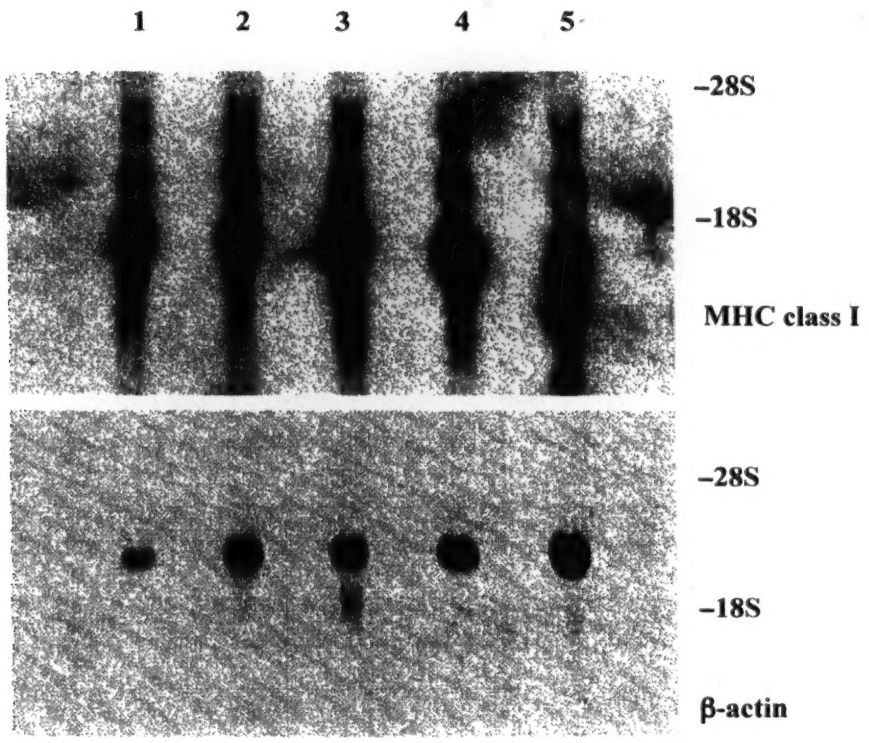

Figure 10: Northern blot analysis of MHC class I expression in spleens from different individuals. Total RNA isolated from the spleens of five ducks was separated, transferred to a blot and hybridized with the MHC class I probe. After probe stripping, the blot was re-hybridized with the β-actin probe. Positions of the 18S and 28S are labeled for reference. 1: duck #95; 2: duck #105; 3: duck #129; 4: duck #132; 5: duck #64.

false results, due to individual variation of the transcriptional level, the expression of these genes should be studied in the same type of tissue coming from the same individual, before and after infection.

3.3.4 Expression of MHC class I genes in ducks congenitally infected with DHBV

The expression of MHC class I genes in ducks congenitally infected with DHBV was assessed and compared with the expression of the same genes in non-infected individuals, through Northern blot assay. Total RNA isolated from the spleens of two healthy individuals, duck #95 and duck #105, non-infected with DHBV, and from the spleens of two congenitally infected individuals, duck #1 and duck #2, was hybridized with the MHC class I probe, under high stringency conditions (Figure 11). The class I probe was stripped and the membrane, re-hybridized with the β-actin probe, under the same conditions. The higher level of MHC class I expression in duck #95 compared with duck #105, observed previously, was confirmed by this experiment. At relatively similar signal intensities for class I, the signal intensity for β-actin was higher in duck #1 in comparison with duck #105, implying that MHC class I genes are expressed at a lower level in duck #1. Sequence analysis of the cDNA transcripts from duck #105 and duck #1 showed that they express different MHC class I alleles. We could not determine whether this difference in class I expression between infected and non-infected individuals, with a magnitude comparable to what we have seen between non-infected ducks, is the result of a DHBV-induced down regulation or of different levels of constitutive MHC class I expression.

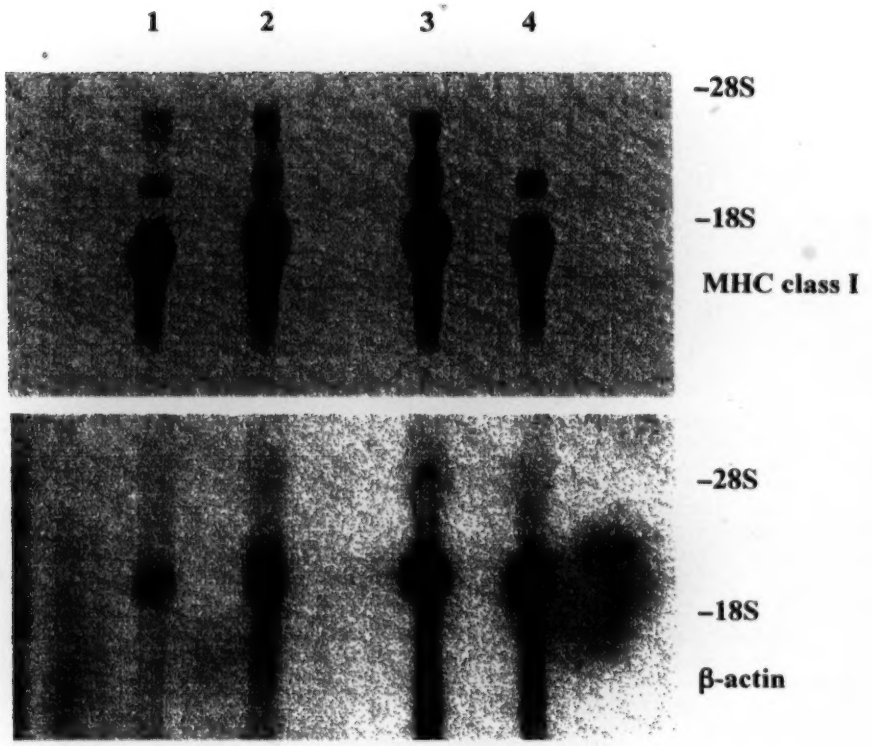

Figure 11: Northern blot analysis of MHC class I expression in spleens from non-infected ducks and ducks infected with DHBV. Total RNA from the spleens of four individuals was separated, blot-transferred and hybridized with the MHC class I probe. Blot was re-hybridized, after probe stripping, with the β-actin probe. Positions of the 18S and 28S are labeled for reference. 1: duck #95 (non-infected); 2: duck #105 (non-infected); 3: duck #1 (DHBV-infected); 4: duck #2 (DHBV-infected).

3.3.5 Expression of MHC class I genes in cultured duck hepatocytes

To investigate the possibility of DHBV interference with the transcription of the MHC class I genes, we cultured primary duck hepatocytes, infected them *in vitro* with DHBV and examined the level of MHC class I expression in total RNA samples collected before and after infection. The primary hepatocytes were obtained from two DHBV-free ducklings, 7 day old, duck #11 and duck #12. Cells were cultured for three days, after which half of the hepatocytes from each individual were infected with DHBV. Both infected and non-infected cells from each duck were harvested on day 3 and day 7 post-infection and the total RNA was hybridized, in Northern blot assays, with the MHC class I, β-actin and DHBV probes, in this order (Figure 12). The plates containing the DHBV-infected cells from duck #11 displayed bacterial contamination at day 7 and were not used for RNA isolation. The stripping of the β-actin probe was not complete, even after multiple cycles, and thus the corresponding band could be seen after hybridization with the DHBV probe and possibly obscured any DHBV bands of similar size. Two hybridizing bands, of approximately 3.5 kb and 5 kb, were visible in the infected hepatocytes samples after using the DHBV probe. The signal intensity was significantly higher at day 7 post-infection, in comparison with day 3, suggesting that the DHBV transcription is increased by cell cycle progression. Previous analysis of the DHBV transcripts in the liver of ducks infected *in vivo* with the virus showed that bands of 3.5 kb, 2.7 kb and 2.5 kb emerged at 12 hours and additional bands, larger than 4 kb, emerged at 24 hours and thereafter (Tagawa et al., 1986). Hybridization with the DHBV probe was the only test that we did to determine whether the *in vitro* infection took place. We did not perform immunostaining to estimate the percentage of cells that were

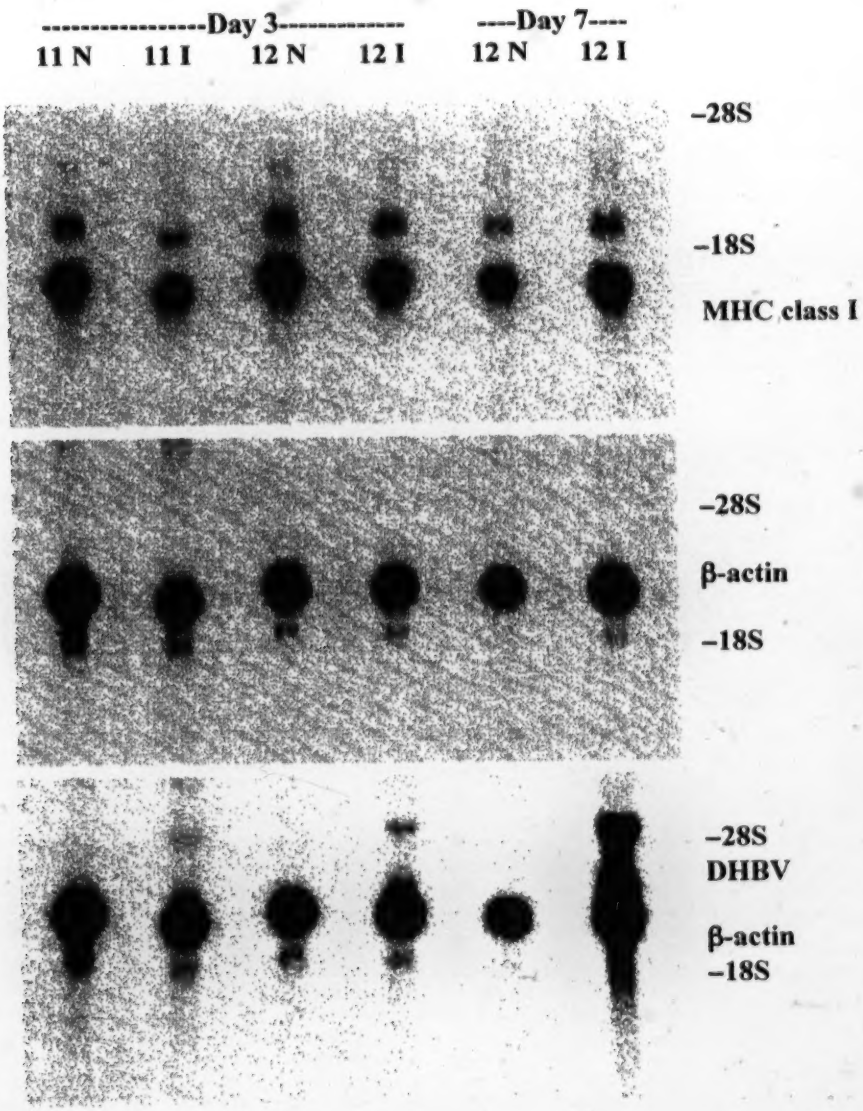

Figure 12: Northern blot analysis of MHC class I, β-actin and DHBV expression in primary duck hepatocytes, cultured *in vitro*. Total RNA isolated from cells, infected and non-infected, at day 3 and day 7 post-infection, was hybridized sequentially with the MHC class I, β-actin and the DHBV probes. Positions of the 18S and 28S are labeled for reference. Abbreviations: 11 N, 12 N, non-infected cells from duck #11 and duck #12, respectively; 11 I, 12 I, DHBV-infected cells from duck #11 and duck #12, respectively.

successfully infected. A visual comparison of the signal intensities corresponding to the MHC class I probe shows that the level of transcription in the infected hepatocytes is the same as in the non-infected counterparts, suggesting that any DHBV interference with the MHC class I expression is post-transcriptional.

4. DISCUSSION

We investigated the allelic diversity and the tissue expression of MHC class I genes in the duck. Cloning and sequencing of the MHC class I transcripts from six ducks showed that each individual expresses two alleles, with the majority of the polymorphic positions being clustered in the exons encoding the peptide binding region. These alleles translate into classical MHC class I molecules with different peptide binding specificities. Amplification of MHC class I from genomic DNA of one duck identified four unique sequences, one of which matched the ones expressed. Northern blot analysis revealed significantly higher MHC class I expression in liver, spleen, intestine and lung in comparison with other tissues and the existence of variability between individuals in the expression of MHC class I. *In vitro* infection of primary duck hepatocytes with DHBV showed that the level of MHC class I gene expression in the infected hepatocytes is the same as in the non-infected counterparts, suggesting that viral replication does not associate with a down regulation of MHC class I transcription.

Homology-based approaches failed to identify duck homologues of the human NKp46 NK cell activating receptor and the chicken CHIR pair of Ig-like receptors. While these receptors of the Ig superfamily retain common features in the organization of their domains, the extensive nucleotide sequence divergence placed them below the limit of detection for PCR amplification and Southern hybridization. An alternative approach to identify these genes would be through the expressed sequence tag (EST) project that is underway in our lab. Sequencing of the cDNA clones and comparison with the sequences in the database would make possible the detection of duck homologues that share less than 70% identity with the human and the chicken genes.

4.1 MHC class I polymorphism in ducks

RT-PCR amplification identified two unique MHC class I sequences in each of the six individuals examined. Three of the ducks came from the same colony as duck #26 and shared the same pair of MHC class I sequences expressed in duck #26, *U*02* and *U*03*, which had been previously identified as alleles at the same locus (Mesa et al., 2004). Duck #26 was the breeding male of the colony, which made us suspect that these ducks were related, although the relationship between these individuals was not known. In total, we identified six different MHC class I sequences in the six ducks examined. Based on the information we had from duck #26 (Mesa et al., 2004), we inferred that the pair of sequences that we found in each duck represent alleles at the dominantly expressed MHC class I locus. Our RT-PCR amplification from six ducks brought thus additional evidence for the dominant expression of one MHC class I gene in ducks and for the codominant expression of the class I alleles at a locus, previously reported in one individual (Mesa et al., 2004). We could not determine whether the overwhelming representation of one allele in each pair (ratios varied between 11/1 and 17/1) is PCR-based or it reflects the relative level of transcription. Differences in the region under the primer sequences could lead to predominant amplification of one sequence, while differences in their promoters could result in biased expression of one allele at the locus. Alignment of the six alleles showed that the majority of the nucleotide substitutions clustered in exon 2 and exon 3, which encode the α1 and α2 domains that form the peptide binding region (PBR). The deduced amino acid sequences corresponding to the six MHC class I alleles displayed the majority of the polymorphic residues in the α1 and α2 domains, among them the α1 domain showing the highest diversity. A higher

diversity in the α1 domain compared with the α2 domain was also observed in the alignments of chicken (Hunt and Fulton, 1998; Livant et al., 2004) and quail (Shiina et al., 1999c) class I protein sequences. The comparatively low diversity in the α3 domain implies selective pressure for polymorphism in the PBR and for conservation in the region contacting the β_2-m and the CD8 co-receptor. Conservation of the positions of high variability found in the human (Parham et al., 1988), chicken (Hunt and Fulton, 1998; Livant et al., 2004), and quail (Shiina et al., 1999c) homologues and of the invariant residues characteristic to classical class I molecules (Shum et al., 1999) strongly suggest that the sequences that we identified encode classical MHC class I glycoproteins with different peptide binding specificities. Our results also suggest that these MHC class I molecules are expressed on the cell surface and are subjected to selective pressure for increased polymorphism. Although the pool of ducks that we examined was relatively small, we have reasons to believe that large, outbred duck populations do not display a limited allelic diversity at the MHC class I locus. The factor that limits the array of peptides that can be presented to CTLs and, consequently, facilitates evasion of immune recognition by the viruses, is thus the expression of one class I locus, despite the presence of additional loci in the genome.

4.2 MHC class I gene expression in hepatocytes infected with DHBV

Our initial strategy to study the DHBV interference with the MHC class I transcription in the duck was to perform Northern blot analysis on liver tissues coming from acutely infected, chronically infected and immune individuals and compare the level of class I expression between these three groups. However, Northern hybridization of total RNA from the spleens of five individuals revealed different levels of MHC class I

constitutive expression. Three of the individuals examined shared the same alleles and, while we expect that identical alleles have identical promoter sequences, differences in the accessibility of the DNA might explain the variability in the transcriptional level. Additionally, Northern blot analysis performed on spleens coming from ducks congenitally infected with DHBV and non-infected individuals showed a difference in the transcriptional level between infected and non-infected tissues of a magnitude comparable to the one seen between non-infected tissues. Therefore, we could not determine whether the difference in gene expression between infected and non-infected individuals was DHBV-related or it represented a variation in the constitutive levels of MHC class I transcription. Examination of different tissues within a DHBV-free individual revealed significantly higher levels of expression of the class I genes in liver, spleen, lung and intestine, compared with other tissues. Given the high level of constitutive expression of these genes in tissues targeted by influenza A and hepatitis B viruses, we predicted that DHBV would down regulate the MHC class I expression in order to establish a persistent infection. *In vitro* infection of primary duck hepatocytes with DHBV and examination of the transcriptional level in non-infected and infected cells from the same individual was therefore our strategy to avoid false results due to variations in the constitutive class I expression between individuals. A comparison between infected and non-infected hepatocytes showed similar levels of gene expression, suggesting that, if there is an effect of DHBV on MHC class I expression, it is post-transcriptional.

In vivo studies of the woodchuck hepatitis virus (WHV) infection showed that acute infection is associated with increased hepatocyte surface expression of the MHC class I

molecules while chronic infection is characterized by an inhibition of the class I display on liver cells (Michalak et al., 2000). In both cases, there was an up-regulation of transcription not only of the MHC class I HC but also of β_2-microglobulin and TAP1 and TAP2 genes induced by IFNγ. The greater than threefold enhancement of intrahepatic transcription of class I HC and β_2-microglobulin genes implies that the defective expression of class I molecules on the surface of chronically infected hepatocytes is due to a viral post-transcriptional effect (Michalak et al., 2000)

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